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Applicant: Hortense DODO *et al.*

Title: DOWN-REGULATION AND SILENCING OF ALLERGEN GENES IN TRANSGENIC PEANUT SEEDS

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Sir:

Transmitted herewith for filing under 37 C.F.R. § 1.53(b) is the nonprovisional utility patent application of:

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Enclosed are:

[X] Specification (65 pages, Claims 1-21 and Abstract (1 pg).

[9] Drawings

[X] Unexecuted Declaration and Power of Attorney (5 pages).

[ ] Assignment of the invention to ________________.

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Respectfully submitted,

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UNITED STATES PATENT APPLICATION

FOR

DOWN-REGULATION AND SILENCING OF ALLERGEN GENES IN TRANSGENIC PEANUT SEEDS

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FIELD OF THE INVENTION

This invention relates to transgenic peanut cells, peanut seeds and peanut products with reduced or undetectable quantities of one or more peanut allergen proteins. The invention also relates to isolated DNA sequences coding for peanut allergen proteins, as well as antisense, genes corresponding to each of the allergen protein genes. Furthermore, the invention relates to recombinant methods of reducing, and eliminating one or more of the peanut allergens in peanut seeds.

BACKGROUND OF THE INVENTION

Food allergy is a serious health problem, and can be life threatening. Public awareness of food allergies is at an all-time high, in part due to the fact that allergic reactions to foods are being reported more frequently. Up to 160 foods have been found to cause allergic reactions (Hefle, 1996, *Crit. Rev. Food Sci. Nutr.* 36:69-89).

The most common allergen-containing foods are peanuts, soybeans, tree nuts, cow's milk, eggs, crustacea, and fish (Taylor, 1992, *Food Technol.*, 148-152; Sampson, 1992. *Food Technol.*, 141-144; and Burks, 1992, *Food Allergy News*, 2:(1) 1). The frequency of food allergy is highest in infancy and early childhood, and decreases with increasing age (Collin-Williams and Levy, 1984, Allergy to food other than milk. In *Food intolerance*, R. K. Chandra, ed., pp. 137-186. Elsevier, New York). About 5% of children younger than three and 1.5% of the general population experience food allergy disorders, or about 4 million Americans suffer from food allergies (Sampson, 1997, *JAMA* 1997, 278 (22): 1888-1894).

Food allergies are increasing worldwide, and peanut is one of the most allergenic food products. It is estimated that over 600,000 children in the United States have peanut allergies. While childhood allergies to egg and cow's milk may disappear, allergies to nuts, peanuts, soybeans, fish and shellfish tend to persist for the lifetime of the individual (Bock, 1982. *J. Allergy Clin. Immunol.*, 69:173-177; Collin-Williams and Levy, 1984, *supra*).

Hypersensitive responses to peanut allergens can be fatal. Contact with the slightest amount of peanut protein can be life threatening to particularly sensitive individuals. There is little data on the incidence of near-fatal and fatal allergic reactions to food, but peanut has been documented as a top offender (Taylor, 1987, *Nutritional Toxicol.* 2: 173-177; Yunginger *et al.*, 1988, *JAMA* 260:(10) 1450-1452; Evans *et al.*, 1988, *CMAJ*, 139: (8) 231-232.; Burks *et al.* 1992; Bock 1992, *J. Allergy Clin. Immunol.*, 90:683-685). It is reported that approximately 125 people die each year in the USA of food-induced anaphylaxis (Burks *et al.*, 1999, *Arch Allergy Immunol*, 119 (3):165-172.)

The allergy can show up at the first exposure to peanuts, often before the age of three. Most people develop peanut allergies early in life, and few ever grow out of peanut allergies, even in adulthood. Allergic reactions to peanuts are often acute and severe (Sampson, 1990. Peanut anaphylaxis. *J. Allergy Clin. Immunol.* 86:1-3). The most common manifestation of peanut allergy is acute hives (or urticaria) following exposure. However, some patients may rapidly develop severe angiodema, swelling of the face, bronchospasm and anaphylaxis, following exposure. Some individuals are so sensitive that they will develop symptoms if they kiss someone who has eaten peanuts or if they eat out of a food utensil that has been in contact with peanuts.

Peanut (*Arachis hypogaea*), a crop grown worldwide, is an annual plant belonging to the family Leguminosae, native to South America, and is commercially grown in the southeastern regions of the United States, specifically in Alabama, Florida, Georgia, North Carolina, and Virginia, and in many other countries of the world. In the United States, several types are grown, although the three most popular peanut types are the Virginia, Spanish, and runner varieties.

Virginia peanuts are used primarily for whole kernel consumption and confections. Runner types are used most frequently for oil production and peanut butter (Woodroof, 1983. In: *Peanuts: Production, Processing, Products*, Woodroof, Ed., Westport, CT). Most of the peanut crop in the United States is used for the production of peanut butter. The most widely cultivated peanut cultivars in the USA are 'Florunner', 'New Mexico Valencia', 'Georgia Green', and 'Georgia Red'.

Although information about the nature and identity of allergenic components of foods is quite limited, it is known that food allergens are most often proteins (Nordlee, *et al.*, 1981., *J. Allergy Clin. Immunol.*, 68:376-383), and provoke an abnormal immunoglobulin E- (IgE)mediated immunological reaction. Several allergenic peanut proteins have been isolated, identified, characterized and classified as minor or major allergens.(Burks AW, Williams LW, Helm RM, Connaughton C, Cockrell G, O'Brien T., *J Allergy Clin Immunol* 1991; 88:172-179; Burks AW, Cockrell G, Connaughton C, Helm RM.; *J Allergy Clin Immunol* 1994; 93:743-750; Gleeson PA, Jermyn MA., *J Plant Physiol* 1977; 4,25; Kleber-Janke T, Crameri R, Appenzeller U, Becker WM, Schlaak M., *Int Arch Allergy Immunol* 1999; 119:265-274; Rabjohn P, Helm EM, Stanley J, West CM, Sampson H, Burks AW, Bannon GA., *J Clin Invest* 1999; 103(4):535-542; Sachs MI, Jones RT, Yunginger JW., *J Allergy Clin Immunol* 1981; 67(1):27-34; Stanley JS., www.ncbi.nlm.nih.gov/htbin...ery?uid=1236995, 1996)

These proteins include glycoproteins, arachin, conarachin, peanut agglutinin and peanut phospholipase. Of these peanut protein allergens, six were classified as major allergens, with an estimated molecular weights of 44, 40, 33, 21, 20, and 18 kDa (De Jong *et al.*, 1998, *Clin. Exp. Allergy*, 28: 743-751).

Burks *et al.* 1992 (*J. Allergy Clin. Immunol. 90: 962-969*) identified two major peanut allergens, designated *Ara h* 1 and *Ara h 2*, which are glycoproteins with isoelectric points and molecular weights of 4.55 and 63,500 Daltons and 5.2 and 17,000 Daltons, respectively. These peanut allergens are stable at a temperature of up to 100 °C, at pH conditions between pH 2.8 and pH 10, and resistant to digestion by acid and digestive enzymes. Peanut, peanut butter,

and peanut flour retain their allergenicity through processing, and crude peanut oil may also be contaminated with these proteins.

The allergens *Ara* h1 and *Ara* h2 are found in the cotyledon of peanut, and both are recognized by more than 90% of peanut-sensitive patients, establishing them as major allergens. *Ara* h1 has been isolated and a cDNA clone produced and sequenced, making this the first peanut allergen to be sequenced (Burks, *et al.*, 1996, *J. Clin. Invest.* 96, 1715-1721). The partial cDNA sequences of *Ara* h2 (Stanley *et al.*, 1997 , *Arch. Biochem. and Biophys,* 342:244-253), *Ara* h3 (Rabjohn, *et al.*, 1999, *J. Clin. Invest.* 103:535-542), *Ara* h4, *Ara* h6, and *Ara* h7 (Kleber-Janke, *et al.*, 1999, *Int. Arch. Allergy Immunol.*, 119:265-274) have also been recently cloned and sequenced.

Currently, no treatment exists for food allergies. Administration of epinephrine and antihistamines is used to reverse the symptoms of food-allergic reactions. Thus, the most effective management strategy in the prevention of peanut allergies is complete avoidance of peanut-containing foods (Schmidl, *et al.*, 1994, *Food Technol.* 10:77-85). However, this is difficult to do, as it requires diligent reading of labels and ingredient listings.

The peanut is a popular and important food, and provides a cheap source of protein and oil for human and animal consumption. Peanuts provide niacin, magnesium, Vitamin C, manganese and chromium in significant amounts and smaller amounts of potassium, Vitamin B6, folic acid, phosphorus, copper and biotin. Furthermore, peanut is widely used in both western and oriental cooking, and is added to a variety of foods such as pastries, sandwiches, egg rolls, chili, syrups, flours, sauces, and confections (Nordlee *et al.*, 1981; Yunginger *et al.*, 1988; Evans *et al.*, 1988; Burks *et al.*, 1991). Because dining out is prevalent in the current American lifestyle, the social stigma associated with refraining from taking part in restaurant or party meals by allergic individuals because of the potential threat for accidentally ingesting peanut, makes the strict avoidance of peanut unlikely and unrealistic (Heiner and Navin, 1975, *J. Allergy Clin. Immunology* 55:82). For example, one of Britain's most promising young athletes died in June 1999, after suffering a seizure triggered by an accidental ingestion of

peanut while eating a chicken sandwich (The Independent -London - June 21, 1999).

An investigation of a wide variety of commercially grown peanuts showed no naturally occurring allergen-free peanut lines. (Dodo HW, Marsic D, Mallender M, Cebert E, Viquez OM. Submitted to *J. Allergy Clin. Immunology,* 2000).

Therefore, there is a need for an alternative solution for the allergic individual. Specifically, there is a need for allergen-free peanut plants, peanuts, and peanut products.

There is also a need for purified peanut allergen proteins which will enable the production of allergen-specific antibodies for detection of allergen in food products, and for prophylaxis and treatment of allergic reactions to peanut.

Modern tools of molecular biology have the potential to offer new transgenic allergen-free peanuts to the peanut-allergic population and the peanut industry. Therefore, an understanding of the molecular structure and regulatory features of the genes is needed to provide needed information for gene silencing and production of allergen-free peanut seeds.

SUMMARY OF THE INVENTION

The present invention provides an isolated polynucleotide molecule comprising a peanut allergen antisense gene, and/or sense gene, or fragment thereof, operably linked to a promoter and a terminator, the promoter and terminator functioning in a peanut cell. In particular, there is provided an isolated polynucleotide molecule comprising the *Ara h*2 peanut allergen antisense gene together with its structural and regulatory features. Furthermore, there is provided a polynucleotide molecule comprising an antisense gene that codes for an RNA molecule that is complementary to, the mRNA molecule coded for by a peanut allergen protein gene selected from the group consisting of *Ara* h1, *Ara* h2, *Ara* h3,

Ara h4, *Ara* h5, *Ara* h6, and *Ara* h7, and any other peanut allergen gene that can induce an allergic reaction in humans.

The present invention further provides the isolated nucleotide sequences of the antisense genes. Seed-preferred promoters, particularly a constitutive promoter, an inducible promoter and a tissue-preferred promoter, are operably linked to the antisense genes, and/or sense genes.

The invention also provides modified transformation vectors such as pCB13, pB1426, pBI436, comprising a polynucleotide molecule having peanut allergen antisense genes, and/or a sense genes, or fragments thereof, operably linked to a promoter and a terminator, the promoter and terminator functioning in a peanut cell.

Yet further provided is a transformed bacterium containing a polynucleotide molecule comprising a peanut allergen antisense gene, and/or a sense gene or fragments thereof, operably linked to a promoter and a terminator, the promoter and terminator functioning in a peanut cell.

The invention also provides a peanut plant cell containing a polynucleotide molecule comprising a peanut allergen antisense gene, and/or a sense gene, or fragment thereof, operably linked to a promoter and a terminator, the promoter and terminator functioning in a peanut cell. Still further provided is a peanut plant containing a cell comprising the polynucleotide molecule having a peanut allergen antisense gene, and/or a sense gene, or fragment thereof, operably linked to a promoter and a terminator, the promoter and terminator functioning in a peanut cell. Yet further, the invention provides a seed produced by the peanut plant containing a cell comprising the polynucleotide molecule having a peanut allergen antisense gene, and/or a sense gene, or fragment thereof, operably linked to a promoter and a terminator, the promoter and terminator functioning in a peanut cell.

The present invention provides methods for producing a transgenic peanut plant with reduced or undetectable allergen protein content in the seed, comprising transforming a recipient peanut plant cell with a DNA construct comprising a peanut allergen antisense gene, and/or a sense gene, or fragment

thereof, regenerating a peanut plant from the recipient cell which has been transformed with the DNA construct, and identifying a fertile transgenic peanut that produces seeds having reduced or undetectable or undetectable allergen protein content. The recipient peanut plant cell may be transformed by biolistic or *Agrobacterium*-mediated methods.

There is also provided a method wherein the recipient peanut plant cell is transformed with a DNA construct comprising an antisense gene, and/or a sense gene, having a nucleotide sequence based on the *Ara* h1, *Ara* h2, *Ara* h3, *Ara* h4, *Ara* h6, or *Ara* h7 genes, or any other allergen genes. Yet further is provided a method wherein the recipient peanut cell is transformed with a DNA construct comprising more than one antisense gene, and/or sense genes.

The invention further provides a method for producing a transgenic peanut plant with reduced or undetectable allergen protein content in the seed, comprising transforming a recipient peanut plant cell with a DNA construct comprising a peanut allergen gene, or fragment thereof, regenerating a peanut plant from the recipient cell which has been transformed the DNA construct, and identifying a fertile transgenic peanut that produces seeds having reduced or undetectable allergen protein content. Still further, the invention provides a method wherein the recipient peanut plant cell is transformed with the polynucleotide by the biolistic method. Yet further, the invention provides a method wherein the recipient peanut plant cell is transformed with the polynucleotide by the *Agrobacterium*-mediated method.

Also provided is a method wherein the recipient peanut plant cell is transformed with a DNA construct comprising a peanut allergen gene, or fragment thereof, that is the *Ara* h1, *Ara* h2, *Ara* h3, *Ara* h4, *Ara* h5, *Ara* h6, *Ara* h7 or any other peanut allergen gene. The invention further provides a method wherein the recipient peanut cell is transformed with a DNA construct comprising more than one peanut allergen gene.

Also provided is a method wherein homologous sequence region between two or more peanut allergen genes is used to down-regulate peanut allergens. A method is provided for producing a transgenic peanut plant with

reduced or undetectable allergen protein content in the seed comprising the steps of identifying a homologous region common to more than one *Ara* h allergen gene; cloning the homologous region in a vector wherein the homologous region is operably linked to a promoter; transforming a recombinant peanut cell with the vector; and identifying a regenerated fertile transgenic peanut plant that produces seed having reduced or undetectable allergen protein content.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows a Southern hybridization of (A) the *Bam* HI digestion pattern of the positive 50kb lambda clone for *Ara h*2 gene (lane 3), Lambda DNA/*Hind* III markers (lane 1), 1 kb DNA step ladder (lane 2); (B) hybridization of an 80-mer labeled probe with a subcloned 12kb *Bam* HI-fragment; and (C) hybridization of an 62-mer labeled probe with a subcloned 6.5 *Bam* HI-fragment (clones 1-6).

Fig. 2 shows the nucleotide and deduced amino acid sequences of peanut allergen *Ara h*2 gene. The figure also shows a putative TATA box, an ATG initiation codon, the first stop codon (TGA), and putative polyadenylation signal (bold). Six additional stop codons are underlined. The deduced polypeptide encoded by the open reading frame has 207 amino acids residues and includes a putative signal peptide of 21 amino acid residues (underlined).

Fig. 3 shows the PCR amplified region (in capital letters) of *Ara h*2 genomic DNA, cloned in transformation vectors (pUC18 and pBI434) in sense and antisense orientations to down-regulate *Ara h*2, *Ara h*6, and *Ara h*7 allergens in peanut. This region is a portion of the sequence homology region between *Ara h*2, *Ara h*6, and *Ara h*7 allergens.

Fig. 4 shows the PCR amplified region (in capital letters) of *Ara h*3 cDNA, cloned in transformation vectors (pUC18 and pBI434) in sense and antisense orientations to down-regulate *Ara h*3, and *Ara h*4 allergens in peanut. This region is a portion of the sequence homology region between *Ara h*3 and *Ara h*4 allergens.

Fig. 5 shows PCR amplified region (in capital letters) of A*ra h*1 P41B cDNA, cloned in transformation vectors (pUC18 and pBI434) in sense and

antisense orientations to down-regulate *Ara h*1 P41B, and *Ara h*1 P17 allergens in peanut. This region is a portion of the sequence homology region between *Ara h*1 P41B and *Ara h*1 P17 allergens.

Fig. 6 is a schematic representation of plasmid constructs to down-regulate peanut allergens in transgenic peanuts.

Fig. 7 shows the PCR amplified region of *Ara h*5 cDNA (shown in bold), cloned in sense and antisense orientations in transformation vectors (pUC18 and pBI434), to down-regulate *Ara h*5 allergen in peanut.

Fig. 8 shows diagrams of the plasmid constructs used in biolistic and *Agrobacterium*-mediated transformation of peanut.

Fig. 9 shows the nucleotide sequence of the Ara h2 promoter upstream of the ATG initiation codon.

DEFINITIONS

As used herein, the term **gene** should be understood to be a full-length DNA sequence encoding a protein or an RNA molecule, as well as a truncated fragment thereof. A gene can be naturally occurring or synthetic.

Marker gene should be understood as a gene encoding a selectable marker (e.g., encoding antibiotic resistance) or a screenable marker (e.g., encoding a gene product which permits detection or transformed cells or plants). The marker gene for the polynucleotide molecule of the present invention can be any nucleotide sequence which codes for a protein or polypeptide which allows transformed cells to be distinguished from non-transformed cells. The marker gene can be, for example, a herbicide resistance gene, an antibiotic resistance gene, a β-glucuronidase (GUS) gene, or a luciferase gene.

A **promoter** is a nucleotide sequence upstream from the transcriptional initiation site and which contains all the regulatory regions required for transcription. Examples of promoters suitable for use in DNA constructs of the present invention include viral, fungal, bacterial, animal and plant-derived promoters capable of functioning in plant cells. The promoter may be selected from so-called constitutive promoters or inducible promoters. If a promoter is an

inducible promoter, then the rate of transcription increases in response to an inducing agent. In contrast, the rate of transcription is not regulated or largely unregulated by an inducing agent, if the promoter is a constitutive promoter.

Examples of suitable inducible or developmentally regulated promoters include the napin storage protein gene (induced during seed development), the malate synthase gene (induced during seedling germination), the small sub-unit RUBISCO gene (induced in photosynthetic tissue in response to light), the patatin gene (highly expressed in potato tubers) and the like. Examples of suitable constitutive promoters include the cauliflower mosaic virus 35S (CaMV 35S) and 19S (CaMV 19S) promoters, the nopaline synthase promoter, octopine synthase promoter, heat shock 80 (hsp 80) promoter and the like. It will be appreciated that the promoter employed in the present invention should be strong enough to control the transcription of a sufficient amount of an antisense RNA molecule to cause an inhibition of expression of a peanut allergen in transformed cells.

A **tissue-preferred promoter** is a DNA sequence that, when operably linked to a gene, directs a higher level of transcription of that gene in a specific tissue than in some or all other tissues in an organism. Examples of such promoters are a stem-specific promoter such as the AdoMet-synthetase promoter (Peleman *et al.*, 1989, *The Plant Cell* 1:81-93), a tuber-specific promoter (Rocha-Sosa *et al.*, 1989, *EMBO J.* 8:23-29). For example, a **seed-preferred promoter** is a DNA sequence that directs a higher level of transcription of an associated gene in plant seeds. Examples of seed-preferred promoters include the seed specific promoter of the USP gene of *Vicia faber* (U.S. Patent No. 5,917,127); the 7S protein promoter of soybean (Bray *et al.*, 1987, *Planta* 172:364-370) and the 2S promoter (Krebbers *et al.*, 1988, *Plant Physiol.* 87:859-866).

A **terminator** is a DNA sequence at the end of a transcribed unit which signals termination of transcription. These elements are 3'-non-transcribed sequences containing polyadenylation signals which act to cause the addition of polyadenylate sequences to the 3' end of primary transcripts. Examples of terminators particularly suitable for use in nucleotide sequences and DNA

constructs of the invention include the nopaline synthase polyadenylation signal of *Agrobacterium tumefaciens*, the 35S polyadenylation signal of CaMV, octopine synthase polyadenylation signal and the zein polyadenylation signal from *Zea mays*.

An **isolated nucleic acid molecule** is a fragment of nucleic acid molecule that has been separated from the nucleic acid of an organism or other natural environment of the nucleic acid. an isolated nucleic acid molecule includes a chemically-synthesized nucleic acid molecule. Other examples of isolated nucleic acid molecule include in vivo or in vitro transcripts of the nucleic acids of the present invention.

Isolated polypeptides are polypeptides not in their naturally occurring form or have been purified to remove at least some portion of cellular or non-cellular molecules with which the proteins are naturally associated. However, the "isolated" protein may be included in compositions containing other polypeptides for specific purposes, for example, as stabilizers, where the other polypeptides may occur naturally associated with at least one polypeptide of the present invention.

The terms "**complementary**" or "**complementarity**" refer to the capacity of purine and pyrimidine nucleotides to associate non-covalently to form partial or complete double stranded nucleic acid molecules. The following base pairs are naturally complementary: guanine (G) and cytosine (C); adenine (A) and thymine (T); and adenine (A) and uracil (U).

Complementary DNA (cDNA) is a single-stranded DNA molecule that is formed from a mRNA template by the enzyme reverse transcriptase. Typically, a primer complementary to portions of an mRNA is employed for the initiation of reverse transcription. Those skilled in the art also use the term "cDNA" to refer to a double-stranded DNA molecule consisting of such a single-stranded DNA molecule and its complementary DNA strand.

The term **expression** refers to the biosynthesis of a gene product. For example, in the case of a structural gene, expression involves transcription of the structural gene into mRNA and the translation of mRNA into one or more polypeptides. In the case of an antisense gene, expression involves transcription of

the antisense DNA into an antisense RNA molecule that is complementary to the sense mRNA.

In eukaryotes, RNA polymerase II catalyzes the transcription of a structural gene to produce mRNA. A DNA molecule can be designed to contain an RNA polymerase II template in which the RNA transcript has a sequence that is complementary to that of a specific mRNA. The RNA transcript is termed an **antisense RNA** and a DNA sequence that codes for the antisense RNA is termed an **antisense gene**. An antisense RNA molecule inhibits the expression of the gene to which it corresponds.

A **vector** is a DNA molecule, such as a plasmid, cosmid, viruses or bacteriophage, that has the capability of replicating autonomously in a host cell. Cloning vectors typically contain a marker gene and one or a small number of restriction endonuclease recognition sites for insertion of foreign DNA sequences without affecting the essential biological function of the vector.

An **expression vector** is a DNA molecule comprising a gene that is expressed in a host cell. Typically, gene expression is placed under the control of certain regulatory elements, including constitutive or inducible promoters, tissue-specific regulatory elements, and enhancers. Such a gene is said to be "**operably linked to**" or "**operatively linked to**" the regulatory elements.

"**Host cell**" refers to any eukaryotic, prokaryotic, or other cell that is suitable for propagating or expressing an isolated nucleic acid that is introduced into the cell by any suitable means known in the art. The cell can be part of a tissue or organism, isolated in culture or in any other suitable form. A **recombinant host** may be any prokaryotic or eukaryotic cell that contains either a cloning vector or expression vector. This term also includes those prokaryotic or eukaryotic cells that have been genetically engineered to contain an isolated gene in the chromosome or genome of the host cell.

A **transgenic peanut plant** is a plant having one or more plant cells that contain a foreign gene. The foreign gene is usually **non-native**, meaning that it is originated from a source other than the host plant and does not share sequence homology to the host genome. The foreign gene may also be **native**, meaning that

it has the nucleotide sequence found in the host. The transgenic plant is made by one of many transformation methods well-known in the art. As used herein, a **fertile transgenic plant** is capable of transmitting a foreign gene to its progeny of further descendants. As used herein, the term **transformation** refers to alteration of the genotype of a host plant by the introduction of native or non-native nucleic acid sequences into the genomes of the plant cell.

Peanut allergen variants, according to the invention, include DNA or protein molecules that resemble, structurally and functionally, the polynucleotide with the sequence of any peanut allergen gene. Peanut allergen genes that can be used for the present invention include *Ara* h1, *Ara* h2, *Ara* h3, *Ara* h4 *Ara* h5, *Ara* h6, *Ara* h7, and any other such genes that are identified and cloned which induce an allergic response in a human.

Hybridizing peanut allergen variants: Nucleic Acid variants within the invention also may be described by reference to their physical properties in hybridization. One skilled in the field will recognize that nucleic acid can be used to identify its complement or homologue, using nucleic acid hybridization techniques. It will also be recognized that hybridization can occur with less than 100% complementarity. However, given appropriate choice of conditions, hybridization techniques can be used to differentiate among DNA sequences based on their structural relatedness to a particular probe. For guidance regarding such conditions see, for example, Sambrook *et al.*, 1989, *Molecular Cloning - A Laboratory Manual,* 2nd ed., Vol. 1-3; and Ausubel *et al.*, 1989, *Current Protocols in Molecular Biology*, Green Publishing Associates and Wiley Interscience, N.Y.

Structural relatedness between two polynucleotide sequences can be expressed as a function of "stringency" of the conditions under which the two sequences will hybridize with one another. Stringent conditions strongly disfavor hybridization, and only the most structurally related molecules will hybridize to one another under such conditions. Conversely, non-stringent conditions favor hybridization of molecules displaying a lesser degree of structural relatedness. Hybridization stringency, therefore, directly correlates with the structural

relationships of two nucleic acid sequences (Bolton *et al.*, 1962, *Proc. Natl. Acad. Sci.* 48:1390.

Hybridization stringency is thus a function of many factors, including overall DNA concentration, ionic strength, temperature, probe size and the presence of agents that disrupt hydrogen bonding. Factors promoting hybridization include high DNA concentrations, high ionic strengths, low temperatures, longer probe size and the absence of agents that disrupt hydrogen bonding.

Hybridization usually is done in two stages. First, in the "binding" stage, the probe is bound to the target under conditions favoring hybridization. A representative hybridization solution comprises 6X SSC, 0.5% SDS, 5X Denhardt's solution and 100μg of non-specific carrier DNA. See Ausubel *et al.*, *supra*, section 2.9, supplement 27 (1994). A stock 20X SSC solution contains 3M sodium chloride, 0.3M sodium citrate, pH 7.0. Of course many different, yet functionally equivalent, buffer conditions are known. For high stringency, the temperature is between about 65 °C and 70 °C in a hybridization solution of 6X SSC, 0.5% SDS, 5X Denhardt's solution and 100μg of non-specific carrier DNA. Moderate stringency is between at least about 40 °C to less than about 65 °C in the same hybridization solution. In both cases, the preferred probe is 100 bases.

Second, the excess probe is removed by washing, which is most important in determining relatedness *via* hybridization. Washing solutions typically contain lower salt concentrations. A medium stringency wash solution contains the equivalent in ionic strength of 2X SSC and 0.5 - 0.1% SDS. A high stringency wash solution contains the equivalent in ionic strength of less than about 0.2X SSC and 0.1% SDS, with a preferred stringent solution containing about 0.1X SSC and 0.1% SDS. The temperatures associated with various stringencies are the same as discussed above for "binding." The washing solution also typically is replaced a number of times during washing. For example, typical high stringency washing conditions comprise washing with 2X SSC plus 0.05% SDS five times at room temperature, and then washing with 0.1X SSC plus 0.1% SDS at 68 °C for 1h. Blots containing the hybridized, labeled probe are exposed to film for one to three days.

Particularly preferred molecules are at least 75 % of the length of those molecules.

Structural variants may also be due to **substitutions, insertions, additions, and deletions**. With regard to amino acid sequence, "substitutions" generally refer to alterations in the amino acid sequence that do not change the overall length of the polypeptide, but only alter one or more amino acid residues, substituting one for another in the common sense of the word. "Insertions," unlike substitutions, alter the overall length of the polypeptide. Insertions add extra amino acids to the interior (not the amino- or carboxyl-terminal ends) of the subject polypeptide. "Deletions" diminish the overall size of the polypeptide by removal of amino acids from the interior or either end of the polypeptide. Preferred deletions remove less than about 30% of the size of the subject molecule. "Additions," like insertions, also add to the overall size of the protein. However, instead of being made within the molecule, they are made on the N- or C- terminus of the encoded protein. Unlike deletions, additions are not very size-dependent. Indeed, additions may be of virtually any size. Preferred additions, however, do not exceed about 100% of the size of the native molecule. The artisan understands "additions" also to encompass adducts to the amino acids of the native molecule.

In general, both the DNA and protein molecules of the invention can be defined with reference to **sequence identity**. As used herein, "sequence identity" refers to a comparison made between two molecules using standard algorithms well-known in the art. Although any sequence algorithm can be used to define "sequence identity," for clarity, the present invention defines identity with reference to the Smith-Waterman algorithm, where the open reading frame of a gene is used as the reference sequence to define the percentage identity of polynucleotide homologues over its length. When "sequence identity" is used with reference to a polypeptide, the entire polypeptide having the sequence of a polypeptide of interest is used as a reference sequence to determine the percent identity of polypeptide homologues over its length.

Preferred polynucleotides are those having at least about 80% sequence identity to the open reading frame. Particularly preferred polynucleotides

have at least about 90% sequence identity. Even more preferred polynucleotides have at least about 95% sequence identity, and most preferred polynucleotides have at least 98% sequence identity. As used herein, two nucleic acid molecules or proteins are said to "share significant sequence identity" if the two contain regions that possess greater than 85% sequence (amino acid or nucleic acid) identity.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

1. Overview

The present invention discloses the isolated, sequenced and characterized genomic clone of the major peanut allergen gene *Ara h 2*. The present invention also provides an isolated polynucleotide molecule comprising the coding sequence for each of the peanut allergen genes operably linked to a promoter and a terminator, the promoter and terminator functioning in a peanut cell. The isolated peanut allergen gene, or fragment thereof, is operably linked to a selected promoter and transformed into peanut cells to make a stably transformed plant.

Peanut seeds comprising multiple copies of a peanut allergen gene exhibit reduced or undetectable allergen protein content due to cosuppression, antisense RNAs, or double-stranded RNAs by combining sense and antisense genes. The selected promoter may be a constitutive or tissue-preferred promoter such as a seed-preferred promoter. Peanut plants may be transformed with more than one peanut allergen gene, or fragment of each gene, in order to produce peanut seeds containing reduced or undetectable quantities of more than one peanut allergen proteins. Alternatively, peanut plants may be transformed with a DNA construct comprising more than one peanut allergen gene, or fragment of each gene, in order to produce peanut plants and seeds containing reduced or undetectable quantities of more than one peanut allergen proteins.

Furthermore, the peanut plants may be transformed with a DNA construct comprising one or more polynucleotide sequences found in more than one peanut allergen gene in a process to produce peanut plants and seeds containing reduced or undetectable quantities of several different peanut allergen proteins. In a preferred embodiment, the peanut allergen gene is selected from the group

consisting of *Ara* h1, *Ara* h2, *Ara* h3, *Ara* h4, *Ara* h5, *Ara* h6, and *Ara* h7, and any other peanut allergen gene, and fragments thereof.

The invention also provides an isolated polynucleotide molecule comprising a peanut allergen antisense gene, and/or a sense gene, and/or combined antisense and sense genes, operably linked to a promoter and a terminator, the promoter and terminator functioning in a peanut cell. The isolated peanut allergen antisense gene, and/or sense gene, and/or combined antisense and sense genes, or fragment thereof, is operably linked to a selected promoter and transformed into peanut cells to make a stably transformed plant. Peanut seeds comprising a peanut allergen gene exhibit reduced or undetectable allergen protein content. The selected promoter may be a constitutive or tissue-preferred promoter such as a seed-preferred promoter.

Peanut plants may be transformed with more than one peanut allergen antisense gene, and/or sense gene, and/or combined antisense and sense genes, or fragment of each gene, in order to produce peanut plants and seeds containing reduced or undetectable quantities of several different peanut allergen proteins. Alternatively, peanut plants may be transformed with a polynucleotide comprising more than one peanut allergen antisense genes, or fragments thereof, in a process to produce peanut plants and seeds containing reduced or undetectable quantities of several different peanut allergen proteins. Furthermore, the peanut plants may be transformed with a DNA construct comprising one or more antisense genes comprising a polynucleotide sequence that is complementary to a DNA sequence found in more than one peanut allergen gene in a process to produce peanut plants and seeds containing reduced or undetectable quantities of several different peanut allergen proteins.

Peanut plants may be transformed with a DNA construct comprising one or more sense genes, comprising a polynucleotide sequence that is similar to a DNA sequence found in more than one peanut allergen genes in a process to produce peanut plants and seeds containing reduced or undetectable quantities of several different peanut allergen proteins.

In a preferred embodiment, the peanut allergen antisense gene generates an RNA molecule which is complementary to a sense mRNA molecule encoding a peanut major allergen protein selected from the group consisting of *Ara* h1, *Ara* h2, *Ara* h3, *Ara* h4, *Ara* h5, *Ara* h6, *Ara* h7, and any other allergen gene, and fragments thereof. The peanut allergen sense gene generates an RNA molecule which is identical to a sense mRNA molecule encoding a peanut major allergen protein selected from the group consisting of *Ara* h1, *Ara* h2, *Ara* h3, *Ara* h4, *Ara* h5, *Ara* h6, *Ara* h7, and any other allergen gene, and fragments thereof. A combination of a peanut allergen antisense gene and sense gene generates a simultaneous expression of sense and antisense sequences corresponding to a peanut major allergen protein selected from the group consisting of *Ara* h1, *Ara* h2, *Ara* h3, *Ara* h4, *Ara* h5, *Ara* h6, *Ara* h7, and any other allergen gene, and fragments thereof.

Also provided is a vector, a bacterium, and a peanut plant cell comprising the polynucleotide molecules of the invention. Still further provided is a method for producing a transgenic peanut plant with reduced or undetectable allergen content. The method comprises a) preparing the polynucleotide molecules of the instant invention; b) transforming a recipient peanut plant cell with the polynucleotide molecules of the instant invention; c) regenerating a peanut plant from the recipient cell which has been transformed with the polynucleotide molecule; and d) identifying a fertile, transgenic peanut plant comprising the polynucleotide molecule and reduced or undetectable allergen content. A preferred embodiment of the method utilizes a biolistic apparatus or a *Agrobacterium* Ti plasmid for the transformation of the peanut plant cell. The polynucleotide molecules of the instant invention include peanut allergen genes, peanut allergen antisense genes, peanut allergen sense genes, and a combination of peanut allergen antisense and sense genes, and fragments thereof.

The present invention also provides methods for testing for allergens in transgenic peanuts using ELIZA.

The present invention also provides methods, utilizing traditional plant breeding procedures, for incorporating the allergen-free peanut germplasm into diverse peanut genetic backgrounds.

2. Peanut Allergen Genes

Peanut contains multiple allergens. An allergen is defined as a molecule that elicits an abnormal immunoglobulin E (IgE)-mediated immunological reaction within certain individuals. Burks *et al.*, 1992, *J. Allergy Clin. Immunol.*, 90: 962-969, identified two glycoproteins which are major peanut allergens, *Ara* h1 and *Ara* h2, with molecular weight and isoelectric points of 63.5 kDa and 4.55 and 17 kDa and 5.2 respectively. De Jong *et al.*, 1998 *Clin. Exp. Allergy*, 28 (6): 743-751, identified and classified six peanut proteins as major allergens with an estimated molecular weight of 44, 40, 33, 21, 20, and 18 kDa. Rabjohn *et al.*, 1999, *J. Clin. Invest.* 103(4): 535-542, isolated another peanut allergen *Ara* h3 Kleber-Janke *et al.*, 1999, *Int. Arch. Allergy Immunol.* 119:265-274, identified and cloned *Ara* h4, *Ara* h5, *Ara* h5, *Ara* h6 and *Ara* h7 by Kleber-Janke T., *et al.*, 1999.

The nucleotide sequences of the published *Ara* clones can be obtained as follows: *Arah*1, Clone P41B (GenBank Accession number L34402), Burks W, Cockrell, Stanley ST, Helm RM and Bannon GA (1995),*Clin. Invest* 96: 1715-1721; *Arah*1 Clone P17 (GenBank Accession number L38853), Burks W, Cockrell, Stanley ST, Helm RM and Bannon, *Unpublished; Arah*2 cDNA (GenBank Accession number L77977),Stanley JS, *Unpublished*; *Arah*2 genomic DNA, Viquez OM, Summer CG and DODO WH (2000), accepted for publication in *The Journal of Allergy and Clinical Immunology; Arah*3 cDNA (GenBank Accession number AF093541), Robinson P, Helm EM, Stanley SJ, West CM, Sampson HA, Burk AW and Banonn GA (1998) *Unpublshed; Arah*4 cDNA (GenBank Accession number AF086821), Kleber-Janke T, Crameri R, Appenzeller U, Schlaak M, and Becker WM (1999), *Int Arch. Allergy Immunol* 119 (4) 265-274; *Arah*5 cDNA (GenBank Accession number AF059616), Kleber-Janke T, Crameri R, Appenzeller U, Schlaak M, and Becker WM (1999), *Int Arch. Allergy Immunol* 119 (4) 265-

274; *Arah6* cDNA (GenBank Accession number AF092846), Kleber-Janke T, Crameri R, Appenzeller U, Schlaak M, and Becker WM (1999), *Int Arch. Allergy Immunol* 119 (4) 265-274; *Arah7* cDNA (GenBank Accession number AF091737), Kleber-Janke T, Crameri R, Appenzeller U, Schlaak M, and Becker WM (1999), *Int Arch. Allergy Immunol* 119 (4) 265-274.

3. Isolation of genes encoding peanut allergen proteins

Several different methods are available for isolating genes coding for peanut allergen proteins. Most approaches begin with the purification of the protein. The purified protein is then subjected to amino acid microsequencing, either directly or after limited cleavage. The partial amino acid sequence that is obtained can be used to design degenerate oligonucleotide probes or primers for use in the generation of unique, nondegenerate nucleotide sequences by polymerase chain reaction (PCR), sequences that can in turn be used as probes for screening genomic DNA libraries. Antibodies raised against purified protein may also be used to isolate DNA clones from expression libraries.

Alternatively, the sequences of DNA coding for related proteins may be used as starting points in a cloning strategy, so-called "cloning by homology". Another way of utilizing sequence information from different species is to take advantage of shorter areas of high sequence homology among related DNAs from different species and to perform PCR to obtain "species-specific" nondegenerate nucleotide sequences. Such a sequence can then be used for library screening or even for direct PCR-based cloning. Detection of the desired DNA can also involve the use of PCR using novel primers.

Libraries are screened with appropriate probes designed to identify the genomic DNA of interest. For expression libraries (which express the protein), suitable probes include monoclonal or polyclonal antibodies that recognize and

specifically bind to the peanut allergen protein. Screening the genomic DNA library with the selected probe may be accomplished as described in the example below.

Screening genomic DNA libraries using synthetic, degenerate oligonucleotides based on partial amino acid sequences, or oligonucleotides based on 5' cDNA sequences, or oligonucleotides based on homologous regions between several allergens of purified known peanut allergen proteins as probes, are the preferred methods of this invention.

The oligonucleotide sequences selected as probes should be of sufficient length and sufficiently unambiguous to minimize false positives. The design of actual nucleotide sequence(s) of the probe(s) is based on regions of the peanut allergen protein that have the least codon redundancy. The oligonucleotides may be degenerate at one or more positions, i.e., two or more different nucleotides may be incorporated into an oligonucleotide at a given position, resulting in multiple synthetic oligonucleotides. The use of degenerate oligonucleotides is of particular importance where a library is screened from a species in which preferential codon usage is not known.

The oligonucleotide can be labeled according to procedures well known in the art, such that it can be detected upon hybridization to DNA in the library being screened. A preferred method of labeling is to use ATP and polynucleotide kinase to radiolabel the 5' end of the oligonucleotide. However, other methods may be used to label the oligonucleotide, including, but not limited to, biotinylation or enzyme labeling.

4. Construction of Nucleic Acids

The isolated nucleic acids of the present invention can be made using (a) standard recombinant methods, (b) synthetic techniques, (c) purification techniques, or combinations thereof, as are well known to those skilled in the art. The nucleic acids may conveniently comprise sequences in addition to a polynucleotide of the present invention. For example, a multi-cloning site comprising one or more endonuclease restriction sites may be inserted into the nucleic acid to aid in isolation of the polynucleotide. Also, translatable sequences

may be inserted to aid in the isolation of the translated polynucleotide of the present invention. For example, a hexa-histidine marker sequence provides a convenient means to purify the proteins of the present invention. Additional sequences that may be inserted include adapters or linkers for cloning and/or expression. Use of cloning vectors, expression vectors, adapters, and linkers is equally well known to those skilled in the art.

The various restriction enzymes disclosed and described herein are commercially and/or available and the manner of use of the enzymes including reaction conditions, cofactors, and other requirements for activity are well known to one of ordinary skill in the art (New England BioLabs, Boston; Life Technologies, Rockville, Md.). Reaction conditions for particular enzymes are preferably carried out according to the manufacturer's recommendation.

A variety of cross-linking agents, alkylating agents and radical generating species as pendant groups on polynucleotides of the present invention can be used to bind, label, detect, and/or cleave nucleic acids using methods and reagents known in the art.

i) Recombinant Methods for Constructing Nucleic Acids

The isolated nucleic acid compositions of this invention can be obtained from biological sources using any number of cloning methodologies known to those of skill in the art. Oligonucleotide probes that selectively hybridize to the polynucleotides of the present invention may be used to identify the desired sequence in a cDNA or genomic DNA library. Isolation of RNA and construction of cDNA and genomic libraries is well known to those of ordinary skill in the art.

ii) Synthetic Methods for Constructing Nucleic Acids

The isolated nucleic acids of the present invention can also be prepared by direct chemical synthesis using the solid phase phosphoramidite triester method (Beaucage and Caruthers, *Tetra. Letts*. 22(20): 1859-1862 (1981)); an automated synthesizer (VanDevanter *et al*., *Nucleic Acids Res*., 12: 6159-6168 (1984)); or the solid support method of U.S. Patent No. 4,458,066. Chemical synthesis generally produces a single stranded oligonucleotide. This may be converted into double stranded DNA by hybridization with a complementary

sequence, or by polymerization with a DNA polymerase using the single strand as a template. One of skill will recognize that while chemical synthesis of DNA is limited to sequences of about 100 bases, longer sequences may be obtained by the ligation of shorter sequences.

iii) Recombinant Expression Cassettes

The present invention further provides recombinant expression cassettes comprising a peanut allergen gene, peanut allergen antisense gene, or a peanut sense gene, or a combination of peanut allergen antisense and sense genes, or fragments thereof, operably linked to transcriptional initiation regulatory sequences that will direct the transcription of the polynucleotide in the intended host cell. Both heterologous and endogenous (native) promoters can be employed to direct expression. These promoters can also be used, for example, in recombinant expression cassettes to drive expression of antisense, sense or a combination of antisense and sense nucleic acids, to reduce or to eliminate peanut allergen content in a desired tissue.

In some embodiments, isolated nucleic acids which serve as promoter or enhancer elements can be introduced in the appropriate position (generally upstream) of a non-heterologous form of a polynucleotide of the present invention so as to up or down regulate expression of a polynucleotide of the present invention. For example, endogenous promoters can be altered in vivo by mutation, deletion, and/or substitution. Suitable promoters include the phage lambda PL promoter, the *E. coli lac*, *trp* and *tac* promoters, the SV40 early and late promoters and promoters of retroviral LTRs, to name a few. Other suitable promoters will be known to the skilled artisan. The expression constructs will further contain sites for transcription initiation, termination and, in the transcribed region, a ribosome binding site for translation. The coding portion of the mature transcripts expressed by the constructs will preferably include a translation initiation (AUG) at the beginning and a termination codon (UAA, UGA or UAG) appropriately positioned at the end of the polypeptide to be translated.

The polynucleotides can optionally be joined to a vector containing a selectable marker for propagation in a host. Such markers include, *e.g.*,

dihydrofolate reductase or neomycin resistance for eukaryotic cell culture and tetracycline, ampicillin or kanamycin resistance genes for culturing in E. *coli* and other bacteria. Representative examples of appropriate hosts include, but are not limited to, bacterial cells, such as E. coli, Streptomyces and Salmonella typhimurium cells; and fungal cells, such as yeast cells.

5. Control of Peanut Allergen Gene Expression

The present invention discloses methods to reduce or eliminate the expression of peanut allergen genes on the basis of antisense, co-suppression, dsRNA technology, and ribozymes.

Plant transformation technologies utilize molecular strategies to down-regulate or to inhibit the expression of endogenous plant genes. These proven strategies have been used to make the allergen-free-peanut plants of the instant invention. They include the antisense RNA strategy, homology dependent gene silencing (HDGS), and the double-stranded RNA method. .

In the antisense RNA strategy (reviewed by Watson CF, and Don Grierson (1992), Antisense RNA in Transgenic Plants, fundamentals and applications. Hiatt *Ed.* p255-281), it is considered that an antisense transcript suppresses gene expression post-transcriptionally by inhibiting RNA processing, transport from the nucleus, and translation, by hybridization with the sense molecules.

Matzke MA, and Matzke AJM (*Plant Physiol.* (1995), 107: 679-685), reviewed the mechanisms involved in homology dependent-gene silencing (HDGS). Double-stranded RNA (dsRNA) is a new tool to suppress gene expression in a number of organisms (Fire et al.,1998 *Nature 391*:806-811; Montgomery et al., 1998 *Proc. Natl. Acad. Sci.* 95:15502-15507 ; Kennerdell and Carthew, 1998 *Cell 95*:1017-1026, Misquitta and Paterson, 1999, *Proc. Natl. Acad. Sci* 96:1451-1426 , Ngo et al., 1998, *Proc. Natl. Acad. Sci.* 95:14687-14692) including plants (Waterhouse et al., 1998, *Proc. Natl. Acad. Sci.* 95:13959-13964). Double-stranded RNA has a very high specificity in suppressing the expression of the gene from which the dsRNA sequence is derived without detectable effect on the expression of

genes unrelated in sequence (Fire et al., 1998, Nature 391:806-811). The molecular mechanisms by which dsRNA generates gene silencing are not well understood yet. It is however speculated that, the gene silencing is a result of a cellular defense to dsRNA formation from nuclear transcripts.

Two classes of HDGS are distinguished by their effect on transcription on the target gene. Examples of transcriptional gene silencing (TGS) are known in which the phenomenon of DNA methylation is the key factor. The promoter of the target gene is methylated, and thereby, becomes inactive. (Brusslan AJ, Karlin-Neumann GA, Huang Lu and Tobin ME (1993), *The Plant Cell* 5: 667-677; Neuhuber F, Park YD, Matzke AJM, Matzke MA, (1994) *Molecular and General Genetics* 244: 230-241) When a transgene integrates into a heavily methylated chromosomal region, it is rapidly silenced. By DNA-DNA interactions, a transgene locus that is silenced can lead to silencing of homologous genes. When the silenced locus is methylated the target locus also becomes methylated.

Double-stranded DNA blocks the activity of genes by artificially providing sense and antisense RNA corresponding to the target gene. Gene silencing by dsRNA is a post-transcriptional process (PTGS). It is demonstrated that triggering of PTGS by direct introduction of foreign RNA requires that both the sense and the antisense strands are provided exogenously, even if a cell already has substantial pool of naturally synthesized sense and antisense RNAs from distinct chromosomal sites, to produce a PTGS response (Ngo et al., 1998; Fire et al., 1998; Waterhouse et al., 1998;) All three strategies, antisense RNA, co-suppression and double-stranded RNA are used in the present invention.

Antisense technology is a versatile approach for controlling expression of endogenous cellular genes and extinguishing cellular gene expression. The principle is to introduce into a cell an RNA or a single stranded DNA molecule complementary to the mRNA of the target gene (the "antisense molecule"). The antisense molecule can base-pair with the naturally occurring corresponding cellular mRNA and prevent its translation. The protocol was originally developed for the control of the gene encoding polygalacturonase during fruit ripening in tomato (Smith *et al.* 1988, *Nature* 334:724-726). Considerable effort has been devoted to

the development of antisense RNA technology for the production of novel plant mutants which have the advantage of being stably inherited (Schuch, 1991, *Soc. Exp. Biol.* 117-127).

Antisense technology, however, has not been applied to peanut. Prior to this invention, there has not been peanut plants or germplasm, whether naturally occurring or genetically engineered, that is partially or completely allergen free. In fact, in an ELISA screen of 32 commercial peanut cultivars by the inventors of the instant invention, no allergen–free cultivar was identified, although a significant difference in allergen level was found among the cultivars.

The present invention provides a nucleotide sequence which is an antisense gene encoding an antisense RNA molecule which has a nucleotide sequence complementary to a sense mRNA molecule that codes for a major peanut allergen protein. This antisense gene is under transcriptional control of a promoter and a terminator, both promoter and terminator capable of functioning in peanut plant cells.

The antisense gene can be of any length provided that the antisense RNA molecule encoded by the antisense gene is sufficiently long to form a complex with a sense mRNA molecule encoding a peanut allergen protein. For the purposes of the description of the present invention, the antisense gene can be from about 50 nucleotides in length up to a length which is equivalent to the full length of the gene. Preferably, the length of the DNA encoding the antisense RNA will be from 100 to 1500 nucleotides. The preferred gene of the present invention is a DNA that codes for an RNA having substantial sequence identity or similarity to the mRNA encoding a peanut allergen protein. Thus the antisense DNA of the present invention may be selected from the group of peanut allergen genes or fragments thereof.

The antisense, sense and the combination of antisense and sense peanut allergen genes may consist of a plurality of subsequences, wherein each subsequence codes for an antisense RNA molecule, a sense RNA molecule and a dsRNA molecule directed to a different peanut allergen gene or a different portion of the same peanut allergen gene. Naturally, the skilled artisan will appreciate that

the subsequences can be adjacent to one another, or noncontiguous, in any order. The invention also provides for a nucleotide sequence that is a variant of the antisense genes described herein.

The present invention discloses a DNA construct comprising the nucleotide sequence according to the invention, as well as a modified transformation vectors comprising the sequence or construct. The vector may be a plasmid or virus. The vector may be the Ti plasmid of *Agrobacterium tumefaciens*. The vector advantageously carries a selectable marker gene. The nucleotide sequence of the invention may code for an mRNA which comprises, in the 5' to 3' direction, (i) a promoter, (ii) at least one peanut allergen antisense gene, and (iii) a terminator. In the DNA construct shown in Fig. 6., the *Arah*2 gene is expressed from its native promoter, and the marker genes are operably linked to the CaMV35s promoter.

Conserved sequences from different allergen genes may be used to down-regulate all known and/or existing allergens (*Arah*1, *Arah*2, *Arah*3, *Arah*4, *Arah*5, *Arah*6, *Arah*7, and any other allergens) in peanut plants. Suppression of expression of more than one allergen gene may be done by introducing multiple copies of a gene or gene fragment into a construct, using sense or antisense homologous regions. The resultant construct contains more than one homologous antisense or sense gene fused in frame and may be used to reduce or eliminate expression of more than one target allergen gene.

Suitable transformation vectors such as pUC 18, pBI426 and modified versions of pBI426 (shown in Fig. 8) are used for carrying out biolistic transformation. Modified versions of pBI434 (Dalta et al., 1991), (also shown in Fig. 8), a binary vector for transformation using *Agrobacterium tumefaciens* (Fig. 6). (See Example 3, below) Transformation vectors carry the transgenes, flanked by a promoter such as the *Arah*2 promoter, or the 35S promoter, and the nopaline synthase terminator. The peanut allergen gene may be portions of the open reading frame (ORF) of peanut allergens *Arah*1, *Arah*2, *Arah*3, *Arah*4, *Arah*5, *Arah*6, *Arah*7, or any other allergen gene.

Different types of transformation cassettes are made to down-regulate peanut allergens. Regions of homology between the nucleotide sequences of

different allergens are PCR amplified from the genomic DNA of *Arah*2. The PCR product is cloned in both antisense and sense orientation into the same transformation vector to produce dsRNA in transformed peanut cells. Or, the antisense construct is used in co-transformation with the construct to produce dsRNA in transformed peanut cells. Another alternative, is to synthesize at least 100 base pair oligonucleotides corresponding to the homology region between the above three allergens to make the antisense and sense transgene constructs.

For control of allergen genes *Arah*3, and *Arah*4 , it is noted that cDNA sequences of these two allergens have 95% homology, shown in capital letters in the sequence in Fig. 4. A portion of two hundred base pairs within the homology region is PCR amplified, and then cloned into the above transformation vectors in sense, or antisense orientation. Alternatively, polynucleotides based on the *Arah*3 and *Arah*4 homologous region may be used for allergen gene control, such as synthesis of 100 base pair oligonucleotides within the region of homology and synthesis of at least 100 base pair oligonucleotides at the 5' end of the cDNA. These oligonucleotides are used in the same way as the PCR products.

Other polynucleotides are also used to down-regulate Arah1. Two different Arah1 clones (*Arah*1 P41B, and *Arah*1 P17) were identified in peanut (Burk *et al*, 1995). The cDNA sequences of these two clones show 96% homology, as highlighted by capital letters in the sequence shown in Fig. 5. A portion of at least two hundred base pairs within the homology region is PCR amplified, and then cloned into transformation vectors in sense, and antisense orientations. Other polynucleotides that can be used to regulate *Arah*1 expression include 100 base pair oligonucleotides within the region of homology, and 100 or more base pair oligonucleotides at the 5' end of the cDNA of each clone.

The *Ara* h5 cDNA sequence does not have any homology with other peanut allergens. Fig. 7. shows the PCR amplified region for the antisense and sense constructs (shown in bold in the sequence) to down-regulate *Arah*5 proteins in peanut plants.

6. Transgenic Peanut Plants

The allergen-free peanut seed according to the present invention may be produced in essentially any of the various transformation methods known to those skilled in the art of plant molecular biology. (See, for example, Wu and Grossman, (Eds.) 1987, *Methods of Enzymology*, Vol. 153, Academic Press, incorporated herein by reference). As used herein, the term "transformation" refers to alteration of the genotype of a host plant by the introduction of non-native or native nucleic acid sequences. Particle bombardment of embryogenic callus, or agrobacterium transformation, are the methods of choice for production of transgenic monocotyledonous plants, but has found widespread application for transformation of dicotyledonous plants as well. (Vasil, 1994, *Plant Mol. Biol.* 25, 925-937). In many cases transformed plant cells may be cultured to regenerate whole plants which can subsequently reproduce to give successive generations of genetically modified plants.

Experiments have shown that foreign genes can be transferred to peanut using *Agrobacterium* mediated transformation (Lacorte *et al.*, 1991, *Plant Cell Reports* 10:354-357. Cheng *et al.*, 1991, *Proc. Amer. Peanut Res. Educ. Soc.*, 23:30.) or microprojectile bombardment (Cheng *et al.*, 1991, *supra*; Ozias-Akins *et al.*, 1993, *Plant Science* 93:185-194). The microprojectile bombardment protocol was reported to produce stably transformed peanut plants.

To commence a transformation process in accordance with the present invention, it is first necessary to construct a suitably modified vector and properly introduce the vector into the plant cell. The details of the construction of the vectors utilized herein are known to those skilled in the art of plant genetic engineering.

For example, the allergen-antisense containing constructs utilized in the present invention can be introduced into plant cells using Ti plasmids, root-inducing (Ri) plasmids, and plant virus vectors. For reviews of such techniques see, for example, Weissbach & Weissbach, 1988, *Methods for Plant Molecular Biology*, Academic Press, N.Y., Section VIII, pp. 421-463; and Grierson & Corey, 1988, Plant Molecular Biology, 2d Ed., Blackie, London, Ch. 7-9, and Florsch *et al.*, *Science* 227:1229 (1985), incorporated herein by reference.

One of skill in the art will be able to select an appropriate vector for introducing the nucleic acid sequences of the invention in a relatively intact state. Thus, any vector which will produce a plant carrying the introduced DNA sequence should be sufficient. Even a naked piece of DNA is expected to be able to confer the properties of this invention, though at low efficiency. The selection of the vector, or whether to use a vector, is typically guided by the method of transformation selected.

For example, a heterologous nucleic acid sequence can be introduced into a plant cell utilizing *Agrobacterium tumefaciens* containing the Ti plasmid. When using an *A. tumefaciens* culture as a transformation vehicle, it is most advantageous to use a non-oncogenic strain of the *Agrobacterium* as the vector carrier so that normal non-oncogenic differentiation of the transformed tissues is possible. It is also preferred that the *Agrobacterium* harbor a binary Ti plasmid system. Such a binary system comprises 1) a first Ti plasmid having a virulence region essential for the introduction of transfer DNA (T-DNA) into plants, and 2) a chimeric plasmid. The chimeric plasmid contains at least one border region of the T-DNA region of a wild-type Ti plasmid flanking the nucleic acid to be transferred. Binary Ti plasmid systems have been shown effective to transform plant cells (De Framond, *Biotechnol.*, 1:262, 1983; Hoekema *et al.*, 1983, *Nature* 303:179.) Such a binary system is preferred because it does not require integration into Ti plasmid in *Agrobacterium*.

Methods involving the use of *Agrobacterium* include, but are not limited to: 1) co-cultivation of *Agrobacterium* with cultured isolated protoplasts; 2) transformation of plant cells or tissues with *Agrobacterium*; or 3) transformation of seeds, apices or meristems with *Agrobacterium*.

In addition, gene transfer can be accomplished by in situ transformation by *Agrobacterium*, as described by Bechtold *et al.*, 1993, *C. R. Acad Sci. Paris* 316:1194. This approach is based on the vacuum infiltration of a suspension of *Agrobacterium* cells.

Alternatively, the allergen antisense gene-containing construct described herein can be introduced into a plant cell by contacting the plant cell

using mechanical or chemical means. For example, nucleic acid can be mechanically transferred by direct microinjection into plant cells utilizing micropipettes. Moreover, the nucleic acid may be transferred into plant cells using polyethylene glycol which forms a precipitation complex with genetic material that is taken up by the cell.

The nucleic acid can also be introduced into plant cells by electroporation (Fromm *et al.*, *Proc. Natl. Acad. Sci., U.S.A.* 82:5824 (1985), which is incorporated herein by reference). In this technique, plant protoplasts are electroplated in the presence of vectors or nucleic acids containing the relevant nucleic acid sequences. Electrical impulses of high field strength reversibly permeabilize plant membranes allowing the introduction of nucleic acids. Electroporated plant protoplasts reform the cell wall, divide and form a plant callus. Selection of the transformed plant cells with the transformed gene can be accomplished using phenotypic markers as described herein above.

Another method for introducing nucleic acid into a plant cell is high velocity biolistic penetration by small particles with the nucleic acid to be introduced contained either within the matrix of small beads or particles, or on the surface thereof (Klein *et al.*, 1987, *Nature* 327:70. Although, typically only a single introduction of a new nucleic acid sequence is required, this method particularly provides for multiple introductions.

Cauliflower mosaic virus (CaMV) may also be used as a vector for introducing heterologous nucleic acid into plant cells (U.S. Pat. No. 4,407,956). The CaMV viral DNA genome is inserted into a parent bacterial plasmid creating a recombinant DNA molecule which can be propagated in bacteria. After cloning, the recombinant plasmid may be re-cloned and further modified by introduction of the desired nucleic acid sequence. The modified viral portion of the recombinant plasmid is then excised from the parent bacterial plasmid, and used to inoculate the plant cells or plants. Plasmids pCB13, pBI426, and pBI434 may also be used as vectors for introducing heterologous nucleic acids into plants. Peanut allergen genes are cloned into these vectors in sense or antisense orientation for single transformations or multiple transformations (co-bombardments). (Chen et al., 1998

Nature Biotechnology 16: 1060-1064; Pawloski, Somers et al., 1996 *Mol Biotechnol* 6:17-30) Using *Agrobacterium* Ti vector-mediated plant transformation methodology, all polynucleotide molecules of this invention can be inserted into peanut genomes after the polynucleotide molecules have been placed between the T-DNA border repeats of suitable disarmed Ti-plasmid vectors (Deblaere, R. *et al.*, 1987, *Methods in Enzymology* 153 277-292). This transformation can be carried out in a conventional manner, for example as described in EP 0116718, PCT publication WO 84/02913 and EPA 87400544.0. The polynucleotide molecule can also be in non-specific plasmid vectors which can be used for direct gene transfer (e.g. de la Pena, A., 1987, *Nature*, 325:274-276).

As indicated above, the polynucleotide molecule according to the instant invention preferably encodes antisense RNAs to all peanut allergen genes, including *Ara* h1, *Ara* h2, *Ara* h3, *Ara* h4, *Ara* h5, *Ara* h6, *Ara* h7, and any other peanut allergen genes.

Alternatively, the skilled person will appreciate that nucleotide sequences as defined herein may be introduced into a peanut cell genome which already comprises one or more antisense, or sense, or a combination of sense and antisense allergen genes. Specifically, the polynucleotide molecule may contain the sequence encoding one of the antisense RNAs, the sense RNA, and sequential transformation (retransformation) may be used to introduce a second polynucleotide molecule comprising a different sense or antisense allergen gene. In this case, an alternative system to select transformants is needed for the second round of transformation. For example, two different selectable marker genes are used in the two consecutive transformation steps. The first marker is used for selection of transformed cells in the first transformation, while the second marker is used for selection of transformants in the second round of transformation. Sequential transformation steps using kanamycin and hygromycin have been described, for example by Sandler *et al.* (1988) and Delauney *et al.* (1988). This retransformation leads to the combined expression of two sense or antisense genes, or some combination thereof, resulting in a transgenic plant that is free of or has reduced or undetectable expression of, more than one allergen. This transformation step can

be repeated until all known peanut allergen genes have been silenced and the resulted transgenic peanut plant is completely free of known existing allergens.

Another alternative is to transform a peanut plant using a polynucleotide molecule containing a sequence encoding one antisense RNA, or one sense RNA, and/or a combination of antisense and sense RNA, and transform another peanut plant using a second polynucleotide molecule containing a sequence encoding the other antisense RNA, sense RNA, and/or a combination of antisense and sense RNA, in a single plant genome through crosses of the two independently transformed peanut plants. It is well-known to a skilled person that the plants, prior to crossing, should be rendered homozygous regarding the transgene through selfing. The first plant should be a plant transformed with a first antisense RNA or a sense RNA, and/or a combination of antisense and sense RNA, or an F1 plant derived thereof through selfing. Selection methods can be applied to the plants obtained from this cross in order to select those plants having the two antisense RNAs genes, sense RNA genes, and/or a combination of antisense and sense RNA genes present in their genome (e.g. by Southern blotting) and blocking the expression of one or more allergens (e.g. by separate ELISA detection). A skilled artisan would recognize that this strategy can be repeated, such that further antisense genes, sense genes, and/or a combination of antisense and sense genes, are introduced sequentially by crossing.

7. Allergen-free Transgenic Peanut: Method to Render the Antisense Transgene Homologous, and Use of Allergen Free Peanut in Traditional Breeding Programs

Allergen-free germplasm may be incorporated in traditional breeding programs for incorporation of this novel trait into desirable peanut genotypes. Methods for producing novel, allergen-free peanut hybrids using the transgenic allergen-free peanut plant of the present invention are known in the art. Each of the following references is incorporated in its entirety, herein, by reference: Moore, 1989, K. M. *et al.*, *J. Heredity* 80(3): 252; Norden, A. J., Peanuts, Culture and Uses. *Am. Peanut Res. and Educ. Soc.*, Stillwater, Okla. (C. T.Wilson ed. 1973);

Norden, A. J. in *Hybridization of Crop Plants* (H. H. Hadley ed. 1980); Norden, A. J., *et al.*, Breeding of the cultivated peanut in *Peanut Science and Technology*, (H. E. Pattee ed. 1992); Norden, A. J. *et al.* Florida Agr. Res. 3:16-18 (1984).

Initially, a homozygous line containing the antisense allergen gene can be obtained, following conventional peanut breeding by self-pollination for a number of generations. This homozygous line may be introgressed into diverse peanut backgrounds in the same, or different market classes by breeding methods known in the art, such as successive selection and inbreeding.

The allergen-free peanut germplasm of the present invention can be introgressed into diverse peanut backgrounds in the same, or different market classes, for example, the runner-type market class (A. hypogaea subsp. hypogaea var. hypogaea botanical type Virginia) as well as the Virginia (A. hypogaea subsp. hypogaea var. hypogaea botanical type Virginia), Peruvian (A. hypogaea subsp. hypogaea var. hypogaea botanical type Peruvian runner), Valencia (A. hypogaea subsp. fastigata var. fastigata botanical type Valencia) and Spanish (A. hypogaea subsp. fastigata var. vulgaris botanical type Spanish) market classes. Peanuts in the runner-type market class are the most commonly used varieties and are found in diverse products such as peanut butter, salted nuts and confectionery products. On the other hand, peanut varieties in the Virginia market class are largely used as salted nuts and in-shell market. The Valencia is largely used in peanut butter while the Spanish type is used in certain niche markets where small round peanuts are needed such as confectionery products and red skin peanuts. Finally, the Peruvian runner market class is grown in certain regions of Mexico.

The allergen-free peanut germplasm of the present invention is introgressed into different peanut backgrounds by conventional methods well know to the skilled artisan in the field of peanut breeding. More specifically, crosses are made according to methods described by Norden, A. J., Peanuts, Culture and Uses, supra.. Am. Peanut Res. and Educ. Soc., Stillwater, Okla. (C. T.Wilson ed. 1973); Norden, A. J. in *Hybridization of Crop Plants* (H. H. Hadley ed. 1980); Norden, A. J., *et al.*, Breeding of the cultivated peanut in *Peanut Science and Technology*, (H. E. Pattee ed. 1992); Norden, A. J. *et al. Florida Agr. Res.* 3:16-18 (1984), the

entirety of each is incorporated by reference. Introgression of the allergen-free characteristic is via the traditional plant breeding cross pollination techniques.

Allergen-free peanut plants may be propagated by planting homozygous seeds and harvesting the crop.

8. Production of Foods Using Allergen-free Peanut

Allergen free peanuts produced according to the instant invention are processed and manufactured into food products using methods well known to a skilled artisan. Allergen-free peanut products are produced using the same standard food processing methods, processing equipment and sanitation practices, as those used in the production of their non-allergen-free counterparts. A skilled artisan would recongize that managing the risk of cross contamination in a food plant producing allergen-free peanut products is critical. If possible the system should be dedicated to producing only allergen-free foods (Beckman and Coult, 1999, *Food Testing & Analysis* 5 (3): 15-17).

9. Protein Expression

Polypeptides of the present invention include naturally purified products, products of chemical synthetic procedures, and products produced by recombinant techniques from a prokaryotic or eukaryotic host, including, for example, bacterial, yeast, higher plant, insect and mammalian cells. Polypeptides of the invention can also include an initial modified methionine residue, in some cases as a result of host-mediated processes.

Purified proteins of the present invention may be used in the treatment of individuals allergic to peanut allergens, for example, via percutaneous specific hyposensitization therapy (see, e.g. Kaneko *et al.*, U.S. Pat. No. 5,951,984) or via oral hyposensitization therapy (Wells *et al.*, 1991, *J. Infect. Dis.*, 8:66; Trentham *et al.*, 1993, *Science*, 261:1727; Weiner, *et al.*, 1993, *Science*, 259:1321).

Using the nucleic acids of the present invention, one may express a protein of the present invention in a recombinantly engineered cell such as bacteria, yeast, insect, mammalian, or preferably plant cells. The cells produce the protein in

a non-natural condition (e.g., in quantity, composition, location, and/or time), because they have been genetically altered through human intervention to do so.

It is expected that those of skill in the art are knowledgeable in the numerous expression systems available for expression of a nucleic acid encoding a protein of the present invention. No attempt to describe in detail the various methods known for the expression of proteins in prokaryotes or eukaryotes will be made.

In brief summary, the expression of isolated nucleic acids encoding a protein of the present invention will typically be achieved by operably linking, for example, the DNA or cDNA to a promoter (which is either constitutive or inducible), followed by incorporation into an expression vector. The vectors can be suitable for replication and integration in either prokaryotes or eukaryotes. Typical expression vectors contain transcription and translation terminators, initiation sequences, and promoters useful for regulation of the expression of the DNA encoding a protein of the present invention. To obtain high level expression of a cloned gene, it is desirable to construct expression vectors which contain, at the minimum, a strong promoter to direct transcription, a ribosome binding site for translational initiation, and a transcription/translation terminator. One of skill would recognize that modifications can be made to a protein of the present invention without diminishing its biological activity. Some modifications may be made to facilitate the cloning, expression, or incorporation of the targeting molecule into a fusion protein. Such modifications are well known to those of skill in the art and include, for example, a methionine added at the amino terminus to provide an initiation site, or additional amino acids (*e.g.,* poly His) placed on either terminus to create conveniently located restriction sites or termination codons or purification sequences.

A. Expression in Prokaryotes

Prokaryotic cells may be used as hosts for expression. Prokaryotes most frequently are represented by various strains of *E. coli;* however, other microbial strains may also be used. Commonly used prokaryotic control sequences

which are defined herein to include promoters for transcription initiation, optionally with an operator, along with ribosome binding site sequences, include such commonly used promoters as the beta lactamase (penicillinase) and lactose (lac) promoter systems (Chang *et al.*, *Nature* 198:1056 (1977)), the tryptophan (trp) promoter system (Goeddel *et al.*, *Nucleic Acids Res*. 8:4057 (1980)) and the lambda derived P L promoter and N-gene ribosome binding site (Shimatake *et al.*, *Nature* 292:128 (1981)). The inclusion of selection markers in DNA vectors transfected in *E. coli* is also useful. Examples of such markers include genes specifying resistance to ampicillin, tetracycline, or chloramphenicol.

The vector is selected to allow introduction into the appropriate host cell. Bacterial vectors are typically of plasmid or phage origin. Appropriate bacterial cells are infected with phage vector particles or transfected with naked phage vector DNA. If a plasmid vector is used, the bacterial cells are transfected with the plasmid vector DNA. Expression systems for expressing a protein of the present invention are available using *Bacillus sp.* and *Salmonella* (Palva, *et al.*, 1983, *Gene* 22: 229-235; Mosbach, *et al.*, 1983, *Nature* 302: 543-545).

B. Expression in Eukaryotes

A variety of eukaryotic expression systems such as yeast, insect cell lines, plant and mammalian cells, are known to those of skill in the art. As explained briefly below, a of the present invention can be expressed in these eukaryotic systems. In some embodiments, transformed/transfected plant cells, as discussed *infra*, are employed as expression systems for production of the proteins of the instant invention.

Synthesis of heterologous proteins in yeast is well known. Sherman, F., *et al.*, 1982, *Methods in Yeast Genetics*, Cold Spring Harbor Laboratory is a well recognized work describing the various methods available to produce the protein in yeast. Two widely utilized yeast for production of eukaryotic proteins are *Saccharomyces cerevisiae* and *Pichia pastoris*. Vectors, strains, and protocols for expression in *Saccharomyces* and *Pichia* are known in the art and available from commercial suppliers (e.g., Invitrogen). Suitable vectors usually have expression

control sequences, such as promoters, including 3-phosphoglycerate kinase or alcohol oxidase, and an origin of replication, termination sequences and the like as desired.

A protein of the present invention, once expressed, can be isolated from yeast by lysing the cells and applying standard protein isolation techniques to the lysates. The monitoring of the purification process can be accomplished by using Western blot techniques or radioimmunoassay of other standard immunoassay techniques.

The sequences encoding proteins of the present invention can also be ligated to various expression vectors for use in transfecting cell cultures of, for instance, mammalian, insect, or plant origin. Illustrative of cell cultures useful for the production of the peptides are mammalian cells. Mammalian cell systems often will be in the form of monolayers of cells although mammalian cell suspensions may also be used. A number of suitable host cell lines capable of expressing intact proteins have been developed in the art, and include the HEK293, BHK21, and CHO cell lines. Expression vectors for these cells can include expression control sequences, such as an origin of replication, a promoter (*e.g.*, the CMV promoter, a HSV *tk* promoter or *pgk* (phosphoglycerate kinase) promoter), an enhancer (Queen *et al.,* 1986, *Immunol. Rev.* 89: 49), and necessary processing information sites, such as ribosome binding sites, RNA splice sites, polyadenylation sites (*e.g.*, an SV40 large T Ag poly A addition site), and transcriptional terminator sequences. Other animal cells useful for production of proteins of the present invention are available, for instance, from the American Type Culture Collection Catalogue of Cell Lines and Hybridomas (7th edition, 1992).

Appropriate vectors for expressing proteins of the present invention in insect cells are usually derived from the SF9 baculovirus. Suitable insect cell lines include mosquito larvae, silkworm, armyworm, moth and *Drosophila* cell lines such as a Schneider cell line (See Schneider, 1987, *J. Embryol. Exp. Morphol.* 27: 353-365.

As with yeast, when higher animal or plant host cells are employed, polyadenlyation or transcription terminator sequences are typically incorporated into

the vector. An example of a terminator sequence is the polyadenlyation sequence from the bovine growth hormone gene. Sequences for accurate splicing of the transcript may also be included. An example of a splicing sequence is the VP1 intron from SV40 (Sprague, *et al.*, 1983, *J. Virol.* 45: 773-781). Additionally, gene sequences to control replication in the host cell may be incorporated into the vector such as those found in bovine papilloma virus type-vectors. Saveria-Campo, M., Bovine Papilloma Virus DNA a Eukaryotic Cloning Vector in *DNA Cloning Vol. II a Practical Approach,* D.M. Glover, Ed., IRL Press, Arlington, Virginia pp. 213-238 (1985).

A peanut allergen protein can be recovered and purified from recombinant cell cultures by well known methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. Most preferably, high performance liquid chromatography ("HPLC") is employed for purification.

The monitoring of the purification process can be accomplished by Western blot techniques, radioimmunoassay, or other standard immunoassay techniques. These methods are described in many standard laboratory manuals, such as Sambrook, *supra*, Chapters 17.37-17.42; Ausubel, *supra*, Chapters 10, 12, 13, 16, 18 and 20.

10. Antibodies of the Invention

Antibodies raised against the proteins and protein fragments of the invention also are contemplated by the invention. In particular, the invention contemplates antibodies raised against the peanut allergen protein *Ara* h2, and variants thereof. Described below are antibody products and methods for producing antibodies capable of specifically recognizing one or more epitopes of the presently described proteins and their derivatives. Antibodies include, but are not limited to polyclonal antibodies, monoclonal antibodies (mAbs), humanized or chimeric antibodies, single chain antibodies including single chain Fv (scFv) fragments, Fab

fragments, $F(ab')_2$ fragments, fragments produced by a Fab expression library, anti-idiotypic (anti-Id) antibodies, epitope-binding fragments, and humanized forms of any of the above.

As known to one in the art, these antibodies may be used, for example, in the detection of a target protein in a food sample. It is important for peanut-sensitive individuals to have a means of recognizing and avoiding peanut-containing products. Unfortunately, peanut allergens have been identified in non-peanut foodstuffs manufactured on common processing equipment that were inadequately cleaned. In general, Keating *et al.*, 1990, *J. Allerg. Clin. Immunol.*, 86: 41-4, which is herein incorporated by reference, describes the use of a radioimmunoassay to detect peanut allergens in food processing materials and finished foods. In one embodiment, the allergen is covalently coupled to a solid phase. The allergen is reacted with a patient serum sample containing both allergen specific and non-specific IgE. The allergen reacts with the specific IgE in the patient sample. After washing away non-specific IgE, radioactively labeled antibodies against IgE are added forming a complex. Then unbound radioactively labeled anti-IgE is washed away. and the radioactivity of the bound complex is measured, for example, in a gamma counter. The more bound radioactivity found, the more specific IgE present in the sample. To classify the test results, patient counts are compared directly with counts of reference sera run in parallel. In another embodiment, the "two-site monoclonal antibody enzyme-linked immunosorbent assay" described in Burks *et al.* (U.S. Pat. No. 5,558,869) is used for the detection of the allergen.

The antibody of the present invention may also be utilized as part of treatment methods. For example, Saint-Remy *et al.*, in U.S. Pat. No. 5,026,545 describes a method for treating allergic reaction via administering to a patient a mixture of allergen-antibody complex.

In general, techniques for preparing polyclonal and monoclonal antibodies as well as hybridomas capable of producing the desired antibody are well known in the art (Campbell, A.M., 1984, *Monoclonal Antibody Technology: Laboratory Techniques in Biochemistry and Molecular Biology*, Elsevier Science

Publishers, Amsterdam, The Netherlands; St. Groth *et al.*, 1980, *J. Immunol. Methods* 35:1-21; Kohler and Milstein, 1975, *Nature* 256:495-497), the trioma technique, the human B-cell hybridoma technique (Kozbor *et al.*, 1983, *Immunology Today* 4:72; Cole *et al.*, 1985, in *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96).

i) Polyclonal Antibodies

Polyclonal antibodies are heterogeneous populations of antibody molecules derived from the sera of animals immunized with an antigen, such as an inventive protein or an antigenic derivative thereof. Polyclonal antiserum, containing antibodies to heterogenous epitopes of a single protein, can be prepared by immunizing suitable animals with the expressed protein described above, which can be unmodified or modified, as known in the art, to enhance immunogenicity. Immunization methods include subcutaneous or intraperitoneal injection of the polypeptide.

Effective polyclonal antibody production is affected by many factors related both to the antigen and to the host species. For example, small molecules tend to be less immunogenic than other and may require the use of carriers and/or adjuvant. In addition, host animal response may vary with site of inoculation. Both inadequate or excessive doses of antigen may result in low titer antisera. In general, however, small doses (high ng to low μg levels) of antigen administered at multiple intradermal sites appears to be most reliable. Host animals may include but are not limited to rabbits, mice, and rats, to name but a few. An effective immunization protocol for rabbits can be found in Vaitukaitis, J. *et al.,* 1971, *J. Clin. Endocrinol. Metab.* 33:988-991.

The protein immunogen may be modified or administered in an adjuvant in order to increase the protein's antigenicity. Methods of increasing the antigenicity of a protein are well known in the art and include, but are not limited to coupling the antigen with a heterologous protein (such as globulin β-galactosidase) or through the inclusion of an adjuvant during immunization. Adjuvants include Freund's (complete and incomplete), mineral gels such as aluminum hydroxide,

surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and *Corynebacterium parvum.*

Booster injections can be given at regular intervals, with at least one usually being required for optimal antibody production. The antiserum may be harvested when the antibody titer begins to fall. Titer may be determined semi-quantitatively, for example, by double immunodiffusion in agar against known concentrations of the antigen. See, for example, Ouchterlony *et al.,* 1973, Chap. 19 in: *Handbook of Experimental Immunology,* Wier, ed, Blackwell. Plateau concentration of antibody is usually in the range of 0.1 to 0.2 mg/ml of serum (about 12 μM). The antiserum may be purified by affinity chromatography using the immobilized immunogen carried on a solid support. Such methods of affinity chromatography are well known in the art.

Affinity of the antisera for the antigen may be determined by preparing competitive binding curves, as described, for example, by Fisher, 1980, Chap. 42 in: *Manual of Clinical Immunology,* second edition, Rose and Friedman, eds., Amer. Soc. For Microbiology, Washington, D.C..

ii) Monoclonal Antibodies

Monoclonal antibodies (MAbs), are homogeneous populations of antibodies to a particular antigen. They may be obtained by any technique that provides for the production of antibody molecules by continuous cell lines in culture or *in vivo*. MAbs may be produced by making hybridomas, which are immortalized cells capable of secreting a specific monoclonal antibody.

Monoclonal antibodies to any of the proteins, peptides and epitopes thereof described herein can be prepared from murine hybridomas according to the classical method of Kohler, G. and Milstein, C., 1975, *Nature* 256:495-497; and U.S. Patent No. 4,376,110 or modifications of the methods thereof, such as the human B-cell hybridoma technique (Kosbor *et al.*, 1983, *Immunology Today* 4:72; Cole *et al.*, 1983, *Proc. Natl. Acad. Sci.* USA 80: 2026-2030), and the EBV-

hybridoma technique (Cole *et al.*, 1985, Monoclonal antibodies and cancer therapy, Alan R. Liss, Inc., pp. 77-96).

In one method a mouse is repetitively inoculated with a few micrograms of the selected protein over a period of a few weeks. The mouse is then sacrificed, and the antibody producing cells of the spleen are isolated.

The spleen cells are fused, typically using polyethylene glycol, with mouse myeloma cells, such as SP2/0-Ag14 myeloma cells. The excess, unfused cells are destroyed by growth of the system on selective media comprising aminopterin (HAT media). The successfully fused cells are diluted, and aliquots are plated to microliter plates where growth is continued. Antibody-producing clones (hybridomas) are identified by detection of antibody in the supernatant fluid of the wells by immunoassay procedures. These include ELISA, as originally described by Engvall, 1980, *Meth. Enzymol.* 70:419, western blot analysis, radioimmunoassay (Lutz *et al.*, 1988, *Exp. Cell Res.* 175:109-124) and modified methods thereof.

Selected positive clones can be expanded and their monoclonal antibody product harvested for use. Detailed procedures for monoclonal antibody production are described in Davis, L. *et al.* 1989, *Basic methods in molecular biology*, Elsevier, New York. Section 21-2. The hybridoma clones may be cultivated *in vitro* or *in vivo*, for instance as ascites. Production of high titers of mAbs *in vivo* makes this the presently preferred method of production. Alternatively, hybridoma culture in hollow fiber bioreactors provides a continuous high yield source of monoclonal antibodies.

The antibody class and subclass may be determined using procedures known in the art (Campbell, 1984, *Monoclonal Antibody Technology: Laboratory Techniques in Biochemistry and Molecular Biology*, Elsevier Science Publishers, Amsterdam, The Netherlands). MAbs may be of any immunoglobulin class including IgG, IgM, IgE, IgA, IgD and any subclass thereof. Methods of purifying monoclonal antibodies are well known in the art.

EXAMPLES

The following examples are given to illustrate the present invention. It should be understood that the invention is not to be limited to the specific conditions or details described in these examples. Throughout the specification, any and all references to publicly available documents are specifically incorporated by reference

EXAMPLE 1. **Isolation and characterization of the genomic clones encoding the peanut allergen genes.**

a) Library screening

To identify the genomic clone of the gene coding for the peanut allergen *Ara h*II, a peanut genomic library constructed in a Lambda Fix II vector (Stratagene Inc, La Jolla, CA) was screened with an 80 base pair oligonucleotide probe. The probe sequence (5'ctagtagccctcgccccttttcctcctcgctgcccacgcatctgcgaggcagcagtgggaactccaaggagacagaaga tg-3') corresponds to nucleotide eleven to ninety-one of a published *Ara h*2 cDNA sequence (GeneBank accession L77197).

Twenty picomoles of the probe was end-labeled with radioactive adenosine 5'-triphosphate, tetra (triethylammonium), salt [gamma 32P] (32P) as described by Ausubel *et al.* (Ausubel F, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K. Short Protocols in Molecular Biology. 3rd ed.: John Wiley & Sons, Inc.; 1995) Fresh *Echerichia Coli (E. Coli)* VCS 257 (300μL of 1x1010 cells/mL) were infected with 10μL of the genomic library (1x103 pfu) for 30 minutes at 37°C in a water bath. Then, 7 mL of top agarose (0.7%) at 47°C were added, mixed and spread onto a pre-warmed (37°C) 150 mm 2xLB agar plate. (Sambrook J, Fritsch EF, Maniatis T. Molecular Cloning: A Laboratory Manual. 2nd ed. New York: Cold Spring Harbor Laboratory Press; 1989) The plaques became visible after an overnight incubation at 37°C.

After plaque formation, the culture dishes were stored for 4 hours at 4°C, blotted on a piece of nylon membrane, denatured (NaOH, 0.5N) and neutralized (Tris-HCl, 1M) according to manufacturer's instructions (NEN Life Science Products, Inc., Boston, MA) and the DNA was crosslinked at 12,000 μjoules of UV energy for 45 seconds (UV Stratalinker 1800, Stratagene). Low stringency prehybridization (at 42°C for 3 hours) and hybridization (at 42°C overnight) were performed in the same solution containing 50% (v/v) formamide + 10% (w/v) SDS + 20% (w/v) dextran sulfate + 1x Denhardt's solution + 10 μg/mL salmon sperm DNA. During hybridization the labeled probe was added to the buffer. Membranes were washed with 2x SSC followed by 2x SSC + 0.1% SDS for 15 minutes at room temperature, air dried, exposed to Kodak XAR-5-X ray film and developed after seven days at -80oC. Positive clones were matched with plaques on the Petri dishes, lifted and stored at 4oC in 1 mL SM media containing a few drops of chloroform to prevent bacterial contamination. (Sambrook *et al.*, *supra*) To confirm true positive clones, a second screening is performed as described above.

b) Purification of putative positive clones

Selected putative positive clones were amplified as described by Sambrook *et al.* (Sambrook *et al.*, *supra*) Lysate stocks of recombinant bacteriophage were prepared by infection of *E. coli* VCS 257 with each putative positive clone. The culture was grown for 6-8 hours at 37°C and 300rpm. Purification of lambda DNA was done using a Lambda kit (Qiagen Inc., Valencia CA) and the DNA was quantified using a fluorometer. (Hoefer Scientific Instruments. TKO 100DNA Mini Fluorometer Instruction Manual 1991)

c) Dot Blot Analysis

Positive clones were confirmed by dot blot analysis using a Bio-Dot SF Microfiltration apparatus (Bio-Rad Laboratories, Inc., Hercules, CA) and the Southern hybridization protocol. (Southern, EM., *J Mol Biol* 1975; 98(3): 503-517) One microgram of each purified DNA was blotted and transferred by capillary action to a nylon membrane. DNA was crosslinked to the membranes at 12,000

μjoules of UV energy for 45 seconds. The membrane was prehybridized at 50oC for a least 4 hours in 6x SSPE + 5x Denhardt's solution + 0.05% (w/v) NaPyrPO4 + 0.5% (w/v) SDS + 100 μg/mL salmon sperm DNA and hybridized at 50oC overnight in 6x SSPE + 1x Denhardt's solution + 0.05% NaPyrPO4 + 0.5% SDS with the same 32P end-labeled probe used to screen the library. Stringent washes were performed at 50oC for 15 minutes each in 6x SSPE + 0.1% (w/v) SDS and 2x SSPE + 0.1% (w/v) SDS. After air drying, the membrane was exposed to Kodak X-Omar AR film at -80oC for two days and autoradiographed.

d) Subcloning

The selected positive lambda clone for *Ara h*2 was subcloned into a pBluescript II SK(+/-) phagemid vector (Stratagene, La Jolla, CA) to facilitate sequencing.

e) Subcloning of a 12 kb fragment into a phagemid vector

The selected positive lambda clone was approximately 50 Kb with an insert fragment of about 16 Kb. The clone was digested with *Bam*H I to release the insert and electrophoresed on a 0.7% agarose gel. Five fragments ranging in size from 5.5, 6.5, 9, 12 and 16 Kb were obtained. After Southern hybridization, only the 12 kb fragment hybridized to the 32P-labeled 80-mer probe. The 12 Kb fragment was then gel purified and subcloned into a pBluescript II SK+ plasmid vector (Figure 1). Sequence analysis revealed that the selected 12kb DNA fragment is truncated at a *Bam*H I restriction site located about 212 nucleotides within the gene.

f) Subcloning of a 6.5 kb fragment into a phagemid vector

A 62 base pair probe (5'-gtgcatgtgcgaggcattgcaacagatc atggagaaccagagcgataggttgcaggggaggc-3') was designed from cDNA sequence downstream from the *Bam*H I site to capture the remaining DNA fragment of the Ara *h*II gene. Of the five fragments obtained after digestion of the 50 kb lambda clone with *Bam*H I, only the 6.5 kb fragment hybridized to this probe. This fragment was subcloned into pBluescript II SK+ plasmid vector and sequenced (Figure 1).

g) Restriction enzyme digestion

For the *Bam*H I digestion, the clone was electrophoresed on a 0.7% agarose gel. Five fragments ranging in size from 5.5, 6.5, 9, 12 and 16 Kb were obtained. After Southern hybridization, only the 12 kb fragment hybridized to the 32P-labeled 80-mer probe, and was then gel purified and subcloned into a pBluescript II SK+ plasmid vector (Figure 1). Sequence analysis revealed that the selected 12kb DNA fragment is truncated at a *Bam*H I restriction site located about 212 nucleotides within the gene.

Restriction enzyme digestion with *Bam*H I was performed at 37°C. Fragments were separated by electrophoresis on a 0.7% agarose gel, and five fragments, 5.5, 6.5, 9, 12 and 16 kb, were obtained. Each fragment was cut from the agarose gel and filtered through a Millipore Ultrafree®-DA filter (Millipore Corp., Bedford, MA) and precipitated in 100% ethanol. The digested pBluescript II vector was dephosphorylated with calf intestinal alkaline phosphatase prior to ligation with the DNA fragments, purified with an equal volume of phenol-chloroform, and precipitated in ethanol and resuspended in one volume of TE buffer (5mM Tris (pH 7.5, 0.1mM EDTA) to a final concentration of approximately 0.1 μg/μL.

h) Ligation

A 2:1 and 3:1 ratio of insert to vector DNA was selected. The ligation reaction was performed at 4°C overnight then at room temperature for three hours. About 20 μL of ultra competent bacteria cells GENEHOGS™ Research Genetics (*E. coli* DH10B) were mixed with 1μL of ligation mixture, electroporated and resuspended in 1 mL of 37°C sterile SOC medium as described in the GENEHOGS™ protocol (Research Genetics, Huntsville, AL). Electroporation was performed using a Bio-Rad Gene Pulser electroporator (Bio-Rad Laboratories, Richmond, CA) with the following settings for a 1mm gap electroporation cuvette (BTX™Genetronics, Inc, San Diego, CA): the field strength at 17kV/cm, the resistor at 200 Ω and the capacitor at 25 μF. Positive colonies were selected by blue-white color selection. (Stratagene Incorporation. Instruction manual: pBluescript®IIExo/Mug DNA Sequencing System. 1999) From each plate, white

positive colonies containing a plasmid with an insert were picked and placed onto 6 mL of LB media supplemented with ampicillin (100 μg/mL) and incubated at 37°C for 16 hours at 300rpm. Plasmid DNA was purified using Qiagen Plasmid Purification kit, digested with *Bam*H I and separated on 0.7% agarose gel to confirm the presence of a plasmid containing an *Ara h*2 insert.

i) Southern Hybridization

Digested DNA fragments were transferred onto a nylon membrane using an alkaline transfer protocol according to manufacturer instructions (Pall, NEN™ Life Science Products, Inc., Boston, MA). The DNA was crosslinked on the membrane as previously described and pre-hybridized at 65 °C for 3 hours in HyperHyb buffer (Research Genetics, Inc., Huntsville, AL). The probe was end labeled with 32P as described in the Fermentas kit (Fermentas Inc., Hanover, MD), added to the hybridization solution and incubated at 65 °C for 3 hours in HyperHyb buffer. The membrane was washed three times at 65 °C for 15 minutes each in 0.1x SSC + 0.1% SDS, rinsed once at room temperature in 1x SSC and exposed to x-ray film (Kodak, Biomax™ MS) at -80°C for three hours and autoradiographed.

j) Sequencing

Purified positive p-Bluescript DNA (0.2μg/μL) were sequenced with ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction kit using AmpliTaq® DNA Polymerase, FS at Research Genetics, Inc. and the University of Alabama in Birmingham (UAB) using T3 and T7 sequencing primers.

k) Sequence analysis

Approximately 1.2 kb of the peanut genomic DNA inser thas been completely sequenced for both the sense and antisense strands, as can be seen in Fig. 2. It has been determined that *Ara h*2 is a gene family and contains iso-forms of the gene. Southern Blot analysis and the difference between the originally characterized cDNA clone and the characterization of the genomic clone of the present invention, is consistent with the existence of multiple genes.

Analysis of the sequence reveals a full length *Ara h*2 gene. Sequence analysis, comparison and homology searches are performed using the BLAST

(Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ., *Nucl Acids Res* 1997; 25:3389-3402), and BLAST 2 sequences tools. (Tatusova TA, Madden TL., *FEMS Microbiol Lett* 1999; 174(2):247-250) Determination of leader sequence is done as described by Grierson and Covey. (Grierson D, Covey SN. Plant Molecular Biology. 2nd ed. New York (NY): Chapman and Hall Publishers; 1988)

As evident from inspection of the sequence shown in Fig. 2, the open reading frame of the gene starts with a initiation codon (ATG) at position 1 and ends with a termination codon (TGA) at position 622. The predicted encoded protein is 207 amino acids long and includes a putative transit peptide of 21 residues.

One putative polyadenylation signal AATAAA is identified at position 951. Six additional putative stop codons are observed downstream of the first termination codon at positions 628 (TGA), 769 (TAA) 901(TAA), 946 (TGA), 967 (TGA) and 982 (TGA). In the promoter region, 5' upstream of the start codon, a putative TATA box, TATTATTA is present at position -72. Comparison of the published cDNA and genomic sequences revealed the absence of an intron.

The location of the initiation codon ATG of *Ara h2* is revealed for the first time. Until now only partial cDNA sequences have been published. (Stanley JS, King N, Burks AW, Huang SK, Sampson H, Cockrell G, Helm RM, West M, Bannon GA. *Arch Biochem Biophys* 1997; 342(2):244-253) The open reading frame of the genomic clone of *Ara h2* is 621 nucleotides long while its cDNA (GeneBank accession L77197) is 492 nucleotides long. A comparison of the 2 sequences reveals that the cDNA sequence is 8 nucleotides short at the 5' region and does not include a start codon. In addition, the two sequences have complete identity from nucleotide 9 to 470 of the genomic clone. However, from nucleotide 471 they diverge with no homology downstream from this region at the nucleotide as well as the amino acid levels.

The termination codon is TGA at position 622. Not only is the termination codon usage different between the genomic (TGA) and the cDNA (TAA) clone but the later also ends 152 bp or 51 amino acids earlier than the

genomic clone. Six additional stop codons are present in the 3' untranslated region at positions 628 (TGA), 769 (TAA), 901 (TAA), 946 (TGA), 967 (TGA) and 982(TGA). It is known that some genes have several termination codons (Grierson & Covey, *supra*), however it is unclear which one is preferentially used. A gene usually undergoes post transcriptional and post translational modifications, which could explain some of the differences between the genomic and cDNA sequences.

A putative polyadenylation signal AATAAA is located at position 951 in the 3' untranslated region of the gene. This signal is identical to the consensus sequence for plants. Polyadenylation signals play key role in the stability and translation of the genetic message and direct the termination of transcription by RNA polymerase II (a functional polyadenylation signal and a downstream transcription 'pause' element are required for efficient pol II transcription termination in fission yeast. *See* Birse C, Proudfoot N., http://genome-www.stanford.edu/Saccharomyces/yeast96/f2021.html; Poly(A) signal controls both transcriptional termination and initiation between the tandem *GAL*10 and *GAL*7 genes of *Saccharomyces cerevisiae*. Greger IH, Proudfoot NJ., *EMBO J* 1998; 17(16):4771-4779).

Fig. 2 shows the deduced polypeptide encoded by the open reading frame which has 207 amino acids residues and includes a putative signal peptide of 21 amino acid residues (Nielsen H, Engelbrecht J, Brunak S, von Heijne G. *Protein Engineering* 1997, 10:1-6.). A signal peptide plays a role in the translocation of a protein from the cytosol to the target organelle within the cell. (Alberts B, Bray D, Lewis J, Raff M, Roberts K, Watson JD. Molecular Biology of the Cell. 3rd ed. New York (NY): Garland Publishing, Inc; 1994) It is typically composed of hydrophobic amino acids such as tryptophan, phenylalanine, valine, leucine and isoleucine that have affinity for membranes of organelles. *Ibid*.

In the proximal region of the promoter, a putative TATA box TATTATTA is present at position –72 with respect to the initiation codon. The consensus signal for plant TATA boxes is TATAT/AA1-328. This is the most conserved sequence for RNA polymerase II-mediated transcription and is important

for positioning the start of transcription. (Alberts *et al. supra*; Ellison K, Messing J., *Biotechnology* 1983; 12:115-139).

The 3' end of the *Ara h2* gene (as shown in Fig. 2, downstream of the stop codon of the gene itself) can be fused, or operably linked, to a heterologous gene for expression of that gene.

EXAMPLE 2. Construction strategy of peanut allergen gene plasmids

Peanut allergen gene plasmids were constructed using expression cassettes containing antisense, and/or sense orientation of allergen genes linked to 35S/Ara h2 promoter and nos terminator, as shown in Fig. 8. Five types of constructs were used for the transformation of peanut tissue. The plasmid constructs pBI426, modified pBI426, and pCB13, were used in biolistic transformation. pBI434modified pBI434 were used in *Agrobacterium*-mediated transformation The Ara h2 promoter is shown in Fig. 9.

PCB13 is used in co-bombardments with modified pBI426 which contains peanut allergen fragments (*Ara h* transgenes), to select transgenic plants. pCB13 contains the 35S promoter, hygromycin gene, and the nos terminator. This cassette is cloned into pUC19.

The plasmid pBI426 (Dalta et al., 1991 *Gene* 101: 239-246) contains a fusion gene (GUS fused to nptII) cloned between *Xba*I and *Sac*I, and driven by the 35S promoter. Its also contains a nontranslatable leader sequence of 50 base pairs from Alfalfa Mosaic Virus. The entire expression cassette is cloned into pUC18.

In the modified pBI426 plasmid, for peanut transformation the gus-nptII fusion gene is replaced with DNA sequences from peanut allergens either as PCR products, or synthetic oligonucleotides. *Ara h* transgenes are clones as *Xba*I/*Sac*I fragments in sense and antisense orientations. For comparative studies, 35S promoter is replaced with *Arah2* promoter (shown in Fig. 9).

For *Agrobacterium*-mediated transformation of peanut, plasmid constructs pBI434 and modified pBI434 were used.

pBI434 is a binary vector derived from pBIN19. The T-DNA between the right border (RB) and the left border (LB) contains the 35S promoter, the 50 base pairs leader sequence from alfalfa mosaic virus, the fusion gene gus-nptII, and the nos terminator.

In the modified pBI434, the GUS gene is replaced by allergen DNA sequences (*Ara h* transgene) as *Xba*I/*Pst*I fragments in sense and antisense orientations. NptII is the selection marker, and transgenic tissues are selected with kanamycin or paromomycin.

EXAMPLE 3. **Methodology used in peanut tissue culture, transformation and regeneration**

Peanut varieties

The most widely cultivated peanut cultivars in the USA, 'Florunner', 'New Mexico Valencia', 'Georgia Green', and 'Georgia Red' can be used in the present method, although a person skilled in the art will realize that the method is applicable to other peanut varieties.

Genetic constructs

Efficient strategies to down-regulate genes in transgenic plants utilize antisense RNA, co-suppression, or double-stranded RNA. All three methods are being used to down-regulate peanut allergens. Transformation vectors used are pUC 18 for biolistic transformation, and modified versions of pBI434 (Dalta et al, 1991), a binary vector for transformation using *Agrobacterium tumefaciens*. Transformation vectors carry the transgenes, flanked by a (the *Arah*2 promoter, shown in Fig. 9, or the 35S promoter) and the nopaline synthase terminator. Transgenes are portions of the open reading frame (ORF) of peanut allergens *Arah*1, *Arah*2, *Arah*3, *Arah*4, *Arah*5, *Arah*6, *Arah*7 genes, and any other peanut allergen genes. Previous studies show homologies between nucleotide sequences of the genomic DNA of *Arah*2, and *Arah*6 and *Arah*7 cDNAs (Viquez et al, 2000, see Fig.3). Also, comparison between *Arah*3 and *Arah*4 cDNA sequences obtained

from the gene bank, shows 95% homology. Different types of transformation cassettes are made to down-regulate peanut allergens.

Transformation cassettes type 1 are used to down-regulate *Arah*2, *Arah*6 and *Arah*7 in transgenic peanut. The homology region between nucleotide sequences of the above three allergen genes (Fig. 3), is PCR amplified from the genomic DNA of *Arah*2. The PCR product is then cloned into the transformation vectors (pBI426 or pBI434) in the antisense orientation for the antisense RNA strategy, or in sense orientation for the co-suppression strategy. The PCR product is also operably linked in frame as an antisense and sense fragment for the dsRNA strategy. Also, the antisense construct and sense construct are used in co-transformation for the dsRNA strategy.

Another alternative is to synthesize at least 100 base pairs oligonucleotides corresponding to the homology region, to be used as for the PCR products.

Fig. 3 shows the nucleotide sequences of the coding region of *Arah*2 genomic DNA (Viquez et al, 2000). The homology region between *Arah*2, *Arah*6 and *Arah*7 is shown in capital letters. This region is amplified by PCR and cloned into the transformation vectors (pUC18, or pBI434). Amplified region to be used for down-regulation of *Arah*2, *Arah*6, *Ara h*7 allergens is shown in capital letters. Alternative methods can be used such as 1) to synthesize at least 100 base pairs oligonucleotides within the region of homology, 2) to synthesize at least 100 base pairs oligonucleotides at the 5' end of the cDNA of each allergen gene. These oligonucleotides are used in the same way as for the PCR products.

Transformation cassettes type 2

Transformation cassettes type 2 are used to down-regulate *Ara h*3, and *Ara h*4 allergens in peanut. The cDNAs of these two allergens have 95% homology (Fig. 4). A portion of two hundred base pairs within the homology region is PCR amplified, and then cloned into the above transformation vectors in sense, and/or antisense orientation.

Fig. 4 shows the cDNA sequence of *Arah*4 (Kleber-Janke, T, 1999). The amplified region to be used for down-regulation of both *Ara h*3 and *Ara h*4

allergens is shown in capital letters. Alternative methods that may be used are 1) to synthesize at least 100 base pairs oligonucleotides within the region of homology, 2) to synthesize at least 100 base pairs oligonucleotides at the 5' end of the cDNA of each allergen gene. These oligonucleotides are used in the same way as for the PCR products.

Transformation vectors type 3

Transformation vectors type 3 are used to down-regulate the two clones of *Ara h*1 (*Ara h*1 P41B, and *Ara h*1 P17) in peanut. The cDNA sequence of these two clones show 96% homology. A portion of at least two hundred base pairs within the homology region is PCR amplified, and then cloned into the above transformation vectors in sense, and/or antisense orientation.Fig. 4 shows the nucleotide sequence of *Ara h*1 41B (Burk et al, 1995). The amplified region to be used for down-regulation of the two clones of *Ara h*1 allergens is shown in capital letters. Alternative methods that may be used are 1) to synthesize at least 100 base pairs oligonucleotides within the region of homology, 2) to synthesize at least 100 base pairs oligonucleotides at the 5' end of the cDNA of each allergen gene. These oligonucleotides are used in the same way as for the PCR products.

Fig. 5. shows the sequence of *Ara h*5 cDNA. This sequence does not have any homology with other allergens. The PCR amplified region for antisense and/or sense constructs are shown in bold to down-regulate *Ara h*5 proteins in peanut plants.

Promoters

Promoters are the key elements for gene expression. Peanut allergens are seed storage proteins. Therefore, to target the synthesis of peanut allergens, a seed specific promoter is essential. The inventive approach has developed its own promoter, the *Arah* 2 promoter, shown in Fig. 9 (*see also* Visqez et al, 2000;which is used to drive RNA transcripts of transgenes cloned into pBI426 and pBI434. In addition, for comparitive studies, the *Arah* 2 promoter shown in Fig. 9 is replaced by the 35S promoter from Cauliflower Mosaic Virus, to compare the degree of downreguation. Whichever of the two types of promoter is used, the promoter is inserted into an expression cassette between HindIII and BglII sites, upstream of

and the *Ara h* transgene PCR amplification product which is inserted between a nontranslatable leader sequence of 50 basepairs from Alfala Mosaic Virus, and a nos terminator.

Selection marker

Plasmid pCB13 lox (see Fig.8) is provided by Ozias-Akin (University of Georgia). This plasmid contains the hygromycin gene, driven by the 35S promoter. This plasmid is used to select transgenic peanut plants. In the case of transformation using *Agrobacterium tumefaciens*, plasmid pBI434 (Dalta et al., 1991; see Fig. 8) contains the neomycin phosphotransferase II gene for selection with paromomycin or kanamycin.

Reporter gene

In some experiments, plasmid pBI426 carrying the GUS gene, driven by the 35S promoter, is used in co-bombardment with allergen construct, to monitor transformation in peanut.

Embryogenic cells of peanut variety Georgia green are co-bombarded with pBI426 (containing the gus gene), and pD2, a modified version of pBI426 plasmid which contains the sense construct of *Ara h*2, replacing the fusion gus-nptII gene. Transient GUS assays show transformation events as dark blue spots. Cultures are in selection medium. Embryos were excised from seeds of peanut cultivars "Florunner" and "Georgia green".

All three types of transformation vectors described above are used in each of the strategies of the present invention for antisense transformation (using antisense constructs), co-suppression (using sense constructs), and the combining of antisense and sense constructs to generate in one transformation vector double stranded RNA fusion transcripts under the control of a single promoter (using either the Ara h2 promoter, or the 35S promoter),Constructs for fusion transcripts are used for both biolistic and agrobacterium-mediated transformation.

Peanut regeneration from somatic embryos

Embryos are excised from seeds of peanut, and are sterilized for 30 min in 20% chlorox (v/v) on a shaker at 130 rpm. Seeds are rinced four times in sterile distilled water, and embryo axes are then separated from cotyledons. Embryo axes are plated on MS medium (MS salts and nutrients (Sigma chemical co, St. Louis) supplemented with picloram (3 mg/l), glutamine (1 mg/l), sucrose (20g/l). pH5.8, before autoclaving. Embryos are cultured, and maintained in dark. After about 3 weeks, proembry masses are formed on explants. They are subcultured every three weeks on the same medium.

Transformation

Embryo cultures are bombarded using a PDS 1000/helium driven apparentus (Bio-rad Laboratories, Herculus, Cal.). Five μg of DNA of each construct (single transformations), or five μg of total DNA (co-transformations) are used for biolistic transformation. Gold particles mixed with the transformation plasmids are accelerated using 1100 PSI pressure under a vacuum of 71 cm mercury. One bombardment is conducted for each transformation experiment. After bombardment, embryos are kept on the same medium for 3 days in dark. They are then transferred in liquid medium supplemented with 20mg/l hygromycin for selection. The liquid medium is refreshed every two weeks. Cultures are maintained in dark with shaking at 130 rpm.

Regeneration

Transgenic structures are multiplied on MS +10 mg/l hygromycin + 1mg/l glutamin and maintained in dark for 2-3 weeks. Cultures are then transferred in light on MS + 3mg/l BAP + 1 mg/l GA3 till embryos geminate. Germinated embryos are then transferred on rooting medium (MS + 0.2 m/l NAA). Hygromycin is also added to confirm transgenics, because non-transgenic plants will not grow.

Peanut regeneration from epicotyls

Sterile embryo axes are cultured on MSTDZ medium (MS medium supplemented with myo-inositol (100 mg/l), sucrose (30g/l), and thidiazuron (2.2 ml of a 1mg/ml stock). Culture media are adjusted to pH5.8, before autoclaving.

Epicotyls are excised from 6 day old germinated embryos by an oblique cut below the cotyledon region, followed by a blunt cut above the root axis.

Transformation

Epicotyl sections are washed in ½ MSI solution (MS salts and nutrients (Sigma chemical co, St. Louis) (2.15g/l and myo-inositol 1g/l,pH5.8 sequentially for three times at 20 min each. Explants are then immersed in a solution of *Agrobacterium* diluted with MSI at OD600=0.5-1. Agro/explant mixture is swirled for ten min. Explants are then blotted on sterile towel paper, and then transferred on MSO (MS medium supplemented with myo-inositol (100 mg/l), sucrose (30g/l) for five days co-culture. After the co-culture period, epicotyl explants are transferred on MSTDZ medium supplemented with 400 mg/l carbenicillin, and 200 mg/l kanamycin (selection) until shoot formation. Putative transgenic shoots are excised for transfer on rooting medium MSTDZ + 50 mg/l kanamycin.

EXAMPLE 4. **Regeneration of transformed peanut and verification of antisense expression and level of down regulation of peanut allergens.**

Transformed epicotyl sections are transferred to MSTDZ media supplemented with 200 mg of kanamycin per liter for selection. All plasmid vectors utilized contain kanamycin resistance genes. Epicotyl explants remain on this media for 2-3 weeks under 16h/8h light/dark and 26 ^{O}C incubation. Plasmid pBI434 contains the kanamycin resistance gene.

Regenerated shoots are excised and placed on MSO supplemented with 200 mg kanamycin per liter for selection. This step is for the detoxification of thidiazuron since this chemical prohibits root formation. Regenerated plants are grown on this media for 2-4 weeks under 16h/8h light/dark and 26 ^{O}C incubation.

Putative transgenic shoots are moved to rooting media (MSO supplemented with 50 mg of kanamycin per liter) for selection. Regenerated plants will remain on this media for 2-4 weeks under 16h/8h light/dark and 26 ^{O}C incubation.

When roots are sufficiently developed (2-5 roots, 2 cm or more in length), plantlets are moved to 1/3 Half Hogland solution (Sigma Chemical Co., St. Louis, MO) for 2-3 weeks to harden them prior to moving into soil.

Transient β-Glucuronidase (GUS) expression is determined 5 days after transformation. The intact explants or regenerated shoots are subjected to GUS histochemical assay (Jefferson *et al.*, 1987, *EMBO J.* 16:2901-2907). Transformation events are dark blue spots.

Radiolabeling of the cDNA or partial genomic clones for *Ara* h1 and *Ara* h2 is performed using random oligonucleotides labeling (Amersham, Arlington Heights, IL) with ^{32}P-dCTP. The labeled probes are used for the detection of stable integration of *Ara* h transgenes into transgenic plants.

Non-transformed controls is analyzed to determine basal levels of each gene in transgenic peanuts. The differences in allergen level of expression between the controls and the transformed peanut plants help in determining the level of downregulation in the transformed plants.

Copy number of transformants is also determined using Southern analysis as described in (Sambrook *et al.*, *supra*). Great variability in the level of gene expression between individual transgenic plant containing the same introduced gene has been reported Rosahl *et al.*, 1987. *EMBO J.* 6:1155-1159. This variability has been ascribed to various factors including gene copy number. A high correlation has been observed between gene copy number and increased gene expression.

An equal amount of digested (*Xba*I/*Sac*I) and undigested (intact) genomic DNA (10 μg per lane) is separated by agarose gel electrophoresis using a 0.8% agarose gel, blotted onto a Hybond N$^+$ membrane (NEN Life Sciences, Boston MA). Hybridization probes were the synthetic 78 nucleotides DNA fragment for *Ara* h1 and the 80 nucleotides DNA fragment for *Ara* h2. Prehybridization, and hybridization is held at 60°C for 2 hours and overnight, respectively. The membrane is washed twice for five minutes with 2X SSC (1X SSC is 0.15 M NaCl plus 0.15 M sodium citrate), 0.1% sodium dodecyl sulfate

(SDS) and twice with 0.2XSSC, 0.2%SDS at 60°C for 15 min. Detection of hybridization patterns is performed by autoradiography. The hybridization pattern is used to determine the copy number of the allergen genes per genome.

Enzyme-linked-immuno-sorbant-assay (ELISA) is performed to detect allergen levels in transgenic peanut plants. Proteins are extracted in a neutral pH phosphate buffer and ELISA conducted as described by Ausubel *et al.*, 1995. In: *Short Protocols in Molecular Biology*. There are currently two commercial Elisa kits on the market for the detection of peanut residues: Neogen Corp. (Lansing, Mich.) and Elisa Technologies (Aluchua, Flor.)

Stable transformed peanut plants having undetectable or reduced or undetectable levels of peanut allergens are selected. Due to the use of Biolistics in the transformation of peanut, multiple copies of each gene are found in multiple locations of the genome, resulting in enhanced down-regulation.

EXAMPLE 5. **Verification of transgene transcripts, and level of down-regulation of peanut allergens**

Radio-labeled probes, with 32P-dCTP of DNA sequences corresponding to the transgenes cloned into the transformation vectors, are used in southern and northern blots to detect stable transformations, copy number of transgenes, and RNA transcripts.

An equal amount of digested (*Xba*I/*Sac*I) and undigested (intact) genomic DNA (10 µg per line) is separated by agarose gel electrophoresis using a 1% agarose gel, blotted onto a Hybond N+ membrane (NEN Life Sciences, Boston MA). Prehybridization, and hybridization are held at 60°C for 2 hours and overnight, respectively. The membrane is washed twice for five min with 2XSSC (1X SSC is 0.15 M NaCl plus 0.15M sodium citrate),0.1% sodium dodecyl sulfate (SDS), and twice with 0.2X SSC, 0.2% SDS at 60°C for 15 min. Detection of hybridization patterns is performed by autoradiography.

Enzyme-linked-immuno-sorbant-assay (ELISA) is performed to detect allergen levels in transgenic peanut plants. Proteins are extracted in a neutral pH phosphate buffer and ELISA conducted as described by Ausubel *et al.*, 1995.*In:*

Short Protocols in Molecular Biology. There are currently two commercial ELISA kits on the market for the detection of peanut residues: Neogen Corp. (Lansing, Mich.) and ELISA Technology (Aluchua, Flor.) Stable transformed peanut plants having undetectable or reduced levels of allergens are selected.

WE CLAIM:

1. A method for producing a transgenic peanut plant with reduced or undetectable allergen protein content in the seed, comprising the steps of:

(a) transforming a recipient peanut plant cell with a DNA construct comprising a peanut allergen antisense gene, or a peanut allergen sense gene, or a combination thereof, or fragments thereof;

(b) regenerating a peanut plant from the recipient cell which has been transformed with the DNA construct; and

(c) identifying a fertile transgenic peanut that produces seeds having reduced or undetectable allergen protein content.

2. The method of claim 1, wherein the peanut allergen gene is selected from the group consisting of *Ara* h1, *Ara* h2, *Ara* h3, *Ara* h4, *Ara* h5, *Ara* h6, and *Ara* h7.

3. The method of claim 1, wherein the recipient cell is transformed by the *Agrobacterium*-mediated method.

4. The method of claim 1, wherein the recipient cell is transformed by the biolistic method.

5. The method according to claim 1, wherein the peanut allergen sense or antisense gene, or a fragment thereof, comprises at least a portion of the nucleotide sequence shown in Figure 2.

6. The method according to claim 1, wherein the peanut allergen sense or antisense gene, or fragment thereof, comprises at least a portion of the nucleotide sequence shown in Figure 3.

7. The method according to claim 1, wherein the peanut allergen sense or antisense gene or fragment thereof, comprises at least a portion of the nucleotide sequence shown in Figure 4.

8. The method according to claim 1, wherein the peanut allergen sense or antisense gene, or fragment thereof, comprises at least a portion of the nucleotide sequence shown in Figure 5.

9. The method according to claim 1, wherein the peanut allergen sense or antisense gene, or fragment thereof, comprises at least a portion of the nucleotide sequence shown in Figure 7.

10. An isolated polynucleotide molecule comprising the peanut allergen antisense gene, or fragment thereof, operably linked to a promoter and a terminator, the promoter and terminator functioning in a peanut cell.

11. The polynucleotide molecule of claim 10, wherein the antisense gene, codes for an RNA molecule that is complementary to the mRNA molecule coded for by a peanut allergen protein gene selected from the group consisting of *Ara* h1, *Ara* h2, *Ara* h3, *Ara* h4, *Ara* h5, *Ara* h6 and *Ara* h7.

12. The polynucleotide molecule according to claim 11, wherein the antisense gene has the nucleotide sequence selected from the group consisting of the nucleotide sequences shown in Figures 3, 4, 5 and 7.

13. The polynucleotide molecule according to claim 10, wherein the promoter is selected from the group consisting of constitutive, inducible and tissue-preferred promoter.

14. The polynucleotide molecule according to claim 13, wherein the promoter is a seed-preferred promoter.

15. A vector comprising the polynucleotide molecule of claim 10.

16. A peanut plant cell comprising the polynucleotide molecule of claim 10.

17. A peanut plant comprising the cell of claim 16.

18. A seed produced by the plant of claim 17.

19. An isolated polynucleotide comprising the promoter of the *Ara h2* gene having the nucleotide sequence shown in Figure 9.

20. An isolated polynucleotide consisting essentially of the nucleotide sequence selected from the group consisting of the nucleotide sequences shown in Figures 3, 4, 5 and 7.

21. A method for producing a transgenic peanut plant with reduced or undetectable allergen protein content in the seed, comprising the steps of

(a) identifying a homologous region common to more than one *Ara* h allergen gene;

(b) cloning the homologous region in a vector modified for peanut transformation, wherein the homologous region is operably linked to a promoter;

(c) transforming a recipient peanut plant cell with the vector; and

(d) identifying a regenerated fertile transgenic peanut plant that produces seeds having reduced or undetectable allergen protein content.

ABSTRACT OF THE DISCLOSURE

An allergen-free transgenic peanut seed is produced by recombinant methods. Peanut plants are transformed with multiple copies of each of the allergen genes, or fragments thereof, to suppress gene expression and allergen protein production. Alternatively, peanut plants are transformed with peanut allergen antisense genes introduced into the peanut genome as antisense fragments, sense fragments, or combinations of both antisense and sense fragments. Peanut transgenes are under the control of the 35S promoter, or the promoter of the *Ara h*2 gene to produce antisense RNAs, sense RNAs, and double-stranded RNAs for suppressing allergen protein production in peanut plants. A full length genomic clone for allergen *Ara h*2 is isolated and sequenced. The ORF is 622 nucleotides long. The predicted encoded protein is 207 amino acids long and includes a putative transit peptide of 21 residues. One polyadenilation signal is identified at position 951. Six additional stop codons are observed. A promoter region was revealed containing a putative TATA box located at position –72. Homologous regions were identified between *Ara h2*, *h*6, and *h*7, and between *Ara h*3 and *h*4, and between *Ara h*1P41B and *Ara h*1P17. The homologous regions will be used for the screening of peanut genomic library to isolate all peanut allergen genes and for down-regulation and silencing of multiple peanut allergen genes.

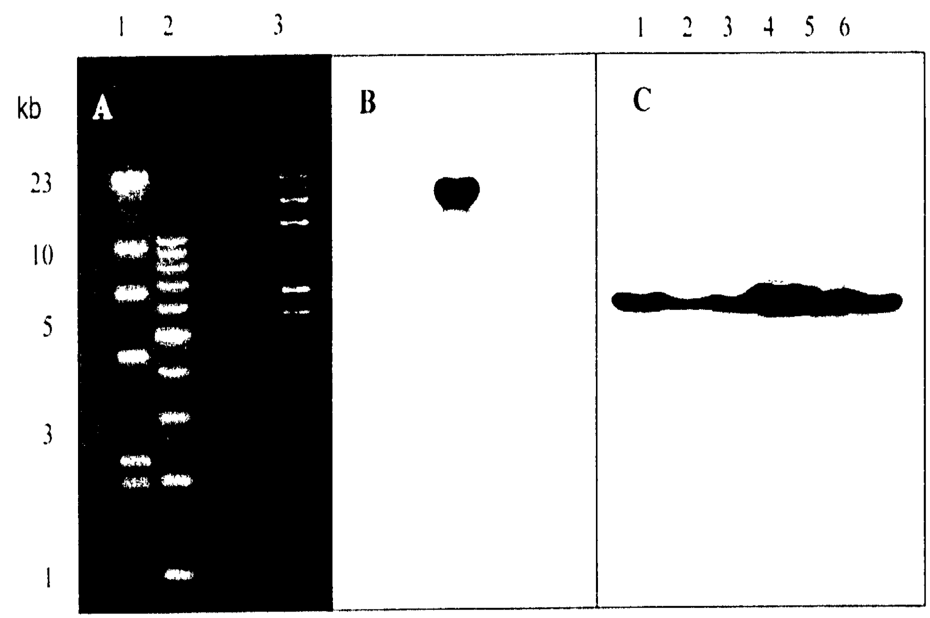

Figure 1. (A) *Bam* HI digestion pattern of positive 50kb lambda clone for *Ara h*II gene (lane3), Lambda DNA/*Hind* III markers (lane1), 1 kb DNA step ladder (lane2). (B) Hybridization of a 80-mer labelled probe with an subcloned 12kb *Bam* HI-fragment. (C) Hybridization of an 62-mer labelled probe with a subcloned 6.5 *Bam* HI-fragment (clones 1-6).

```
                                         tccttacgcgaaatacggg
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Figure 2

Fig. 3.

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Fig. 4.

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Fig. 5.

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Figure 6: Gene constructs for down-regulating peanut allergens in transgenic peanuts.

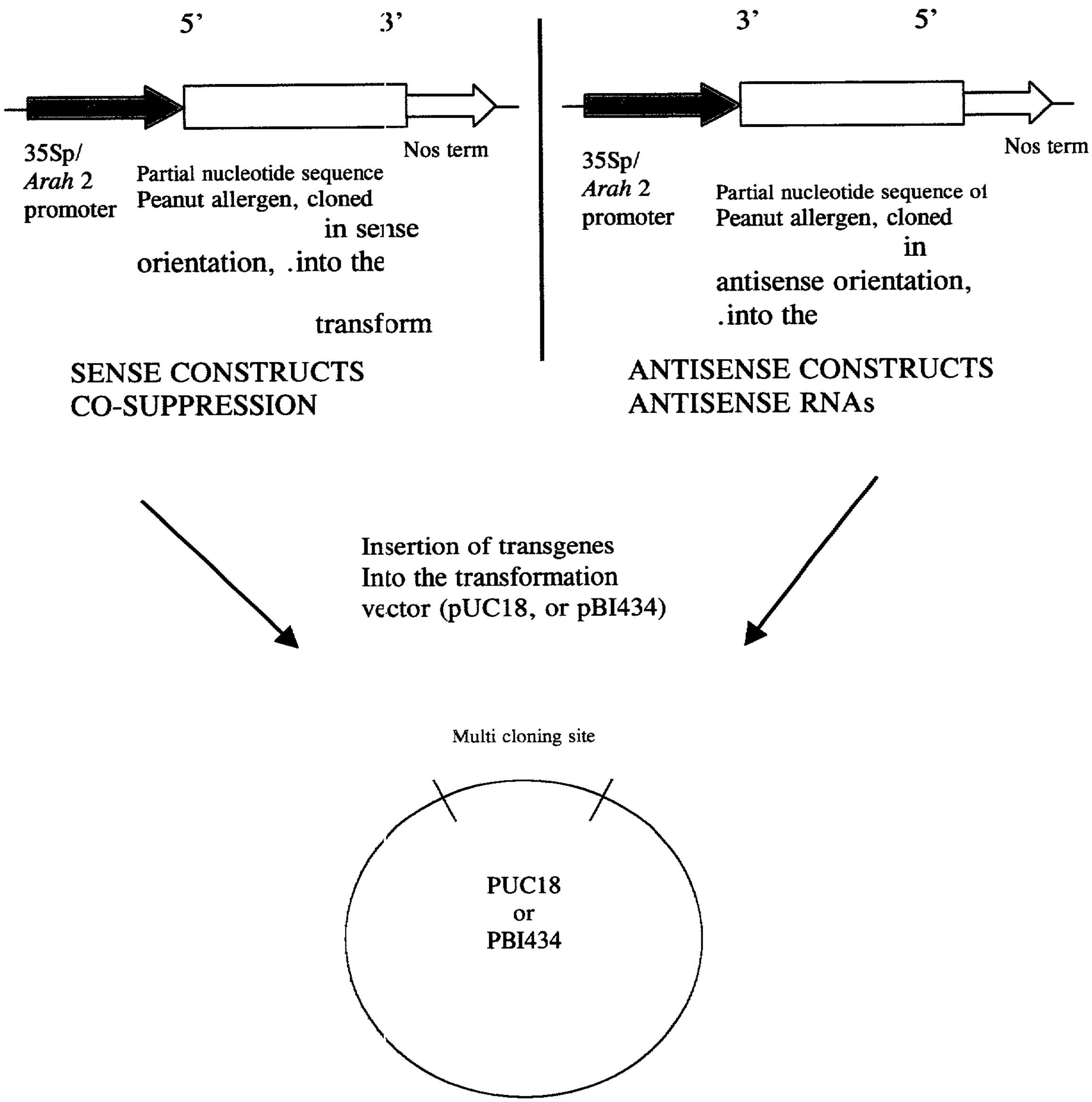

Figure 7.

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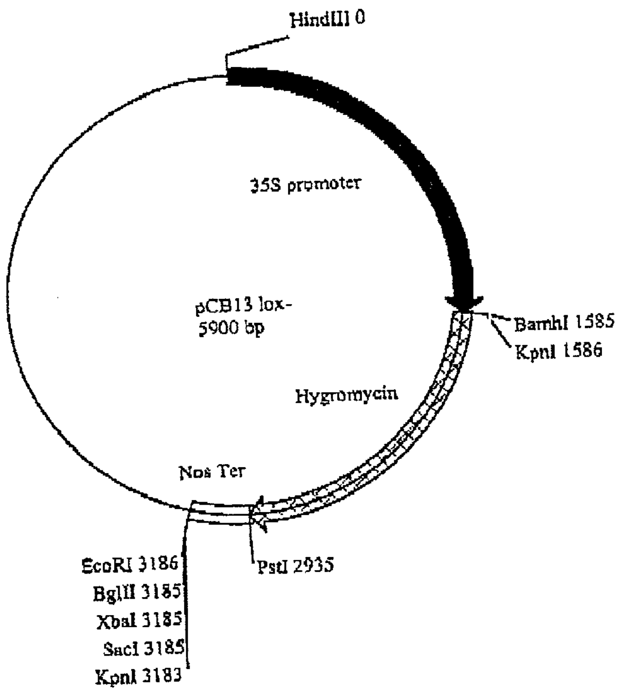
Fig. 8

pCB13 for selection of transgenic plants



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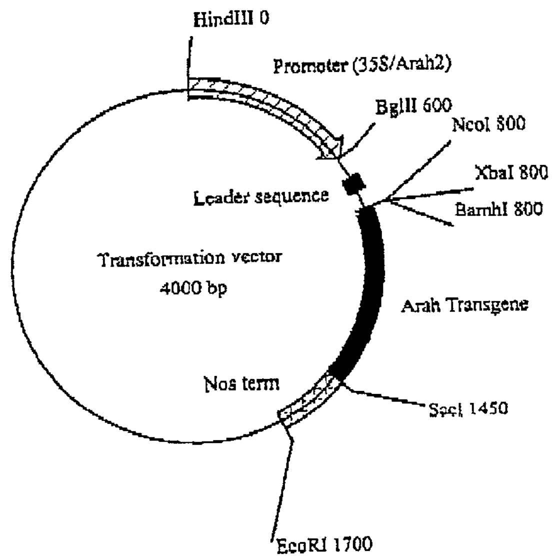

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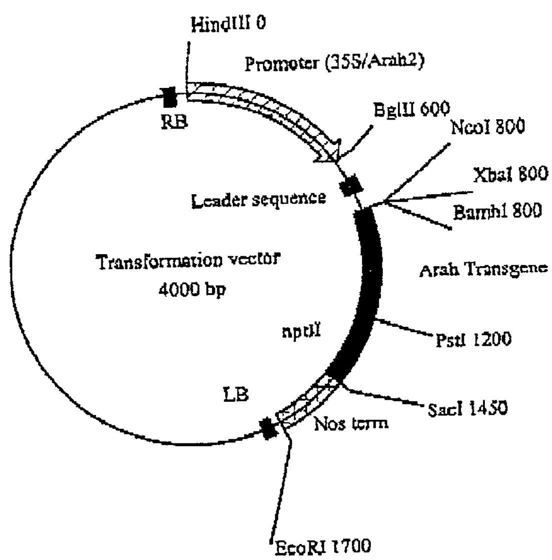

Fig. 9

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  1 atggccaagctcaccatactagtagccctcgcccttttcctcctc
```

Fig. 9 shows the nucleotide sequence of the *Arah2* promoter upstream of the ATG initiation codon of the genomic *Arah2* clone.